Kewal K. Jain

Applications of Biotechnology in Oncology

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ISBN 978-1-4614-9244-3 ISBN 978-1-4614-9245-0 (eBook)
DOI 10.1007/978-1-4614-9245-0
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2013953290

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Printed on acid-free paper

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Preface

This book puts together excerpts from various writings by the author on biotechnology topics as they relate to cancer. It is meant for oncologists, scientists involved in research on cancer biology, and physicians in various specialties who deal with cancer. It will be useful for those working in life sciences and pharmaceutical industries, particularly those involved in the discovery of anticancer agents and drug delivery in cancer. Basics of various "omics" technologies and their application in oncology are described as oncogenomics and oncoproteomics. Because the book is organized according to technologies, only examples of application in cancers of various organs/systems are described in various chapters. This may involve some repetition of overlapping technologies for genomics, proteomics, biomarkers, and molecular diagnostics.

Molecular diagnostics have important applications in the investigation and treatment of cancer. Biomarkers also provide important information relevant to diagnosis, prognosis, and monitoring of treatment. Sequencing is making important contributions to molecular diagnostics. Next generation sequencing has helped to advance our understanding of pathophysiology of cancer, improve molecular diagnosis of cancer, and facilitate anticancer therapeutics.

Nanobiotechnology is making important contributions to cancer diagnosis, drug delivery, and therapy, justifying the use of "nanooncology" as a chapter heading. Many of the drugs developed for cancer failed because of lack of penetration to the site of action in the brain. Routes of drug delivery and applications to various types of cancer are described. Various methods of improving systemic administration of drugs for targeted delivery to cancer are described, including the use of nanobiotechnology.

Cell and gene therapies, including antisense and RNA interference as well as vaccines, have emerged as effective methods for the treatment of cancer. Modern cancer therapies have many biotechnology-based products. Nanobiotechnology is probably the most important biotechnology in relation to the refinement of drug delivery, refinement of molecular diagnostics, and its integration with therapeutics.

Finally, a chapter on personalized oncology is important for the era of personalized medicine. This concept is the best way of integrating new technologies to select the appropriate strategy for the treatment of an individual patient.

Knowledge in biotechnology and oncology has grown tremendously and both fields require massive encyclopedias for coverage. As a handy volume integrating both of these fields, only coverage of selected highlights is possible. The bibliography includes a total of ~1,000 selected references from recent literature on this topic, which are appended to each chapter according to their relevance. The text is supplemented by 53 tables and 10 figures.

The author wishes to acknowledge the help, guidance, and encouragement received during the preparation of this work from David Casey, Editor for this project at Springer, and Patrick J. Marton, Managing Editor, Springer Protocols.

Basel, Switzerland Kewal K. Jain

About This Book

This book contains excerpts from various biotechnology books and reports authored by Prof. K.K. Jain that are relevant to cancer. The most important contributions of biotechnology are to the molecular diagnosis of cancer and drug delivery in cancer for personalized management of patients.

Author's Biography

Professor K.K. Jain is a neurologist/neurosurgeon by training and since his retirement from neurosurgery has been working in the biotechnology/biopharmaceuticals industry as a consultant at Jain PharmaBiotech. He received graduate training in Europe as well as North America and has held academic positions in several other countries. Currently, he is a Fellow of the Royal Australasian College of Surgeons and a Fellow of the Faculty of Pharmaceutical Medicine of the Royal College of Physicians of UK.

Prof. Jain's 448 publications include 24 books (5 as editor + 19 as author) and 50 special reports, which have covered important areas in biotechnology, oncology, cell/gene therapy, and biopharmaceuticals. His "Textbook of Gene Therapy" was translated into Chinese language in 2000. The "Textbook of Hyperbaric Medicine" (5th Ed 2009), which contains a chapter on cancer, has been a standard reference on the subject for the past two decades. Prof. Jain is editing 2nd edition of "Drug Delivery Systems" (Humana/Springer 2014). His recent books include "Textbook of Personalized Medicine" (Springer 2009; Japanese edition 2012), "Handbook of Biomarkers" (Springer 2010), Handbook of Neuroprotection (Springer 2011), and "Handbook of Nanomedicine, 2nd Ed" (Springer/Humana 2012).

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Abbreviations

2DGE	Two-dimensional gel electrophoresis
AAV	Adeno-associated virus
ACT	Adoptive cell therapy
Ad	Adenovirus
ADC	Antibody drug conjugate
ADEPT	Antibody-directed enzyme prodrug therapy
AML	Acute myeloid leukemia
AMP	Adenosine monophosphate
APL	Acute promyelocytic leukemia
ASC	Adult stem cell
ATP	Adenosine triphosphate
BBB	Blood–brain barrier
bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BMT	Bone marrow transplantation
cDNA	Complementary DNA
CEA	Carcinoembryonic antigen
CGH	Comparative genomic hybridization
CHO	Chinese Hamster Ovary
CHOP	Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CMML	Chronic myelomonocytic leukemia
CMV	Cytomegalovirus
CNA	Circulating nucleic acid
CNV	Copy number variation
CRC	Colorectal cancer
CSC	Cancer stem cell
CSF	Cerebrospinal fluid
CTC	Circulating tumor cell

CTLs	Cytotoxic T lymphocytes
DC	Dendritic cell
DDS	Drug delivery system
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EGF	Epidermal growth factor
EPC	Endothelial progenitor cell
ESC	Embryonic stem cell
FANC	Fanconi anemia complementation group
Fas	Apoptosis-stimulating fragment
FDA	Food and Drug Administration, USA
FFPE	Formalin-fixed paraffin-embedded
FGF	Fibroblast growth factor
FGFR	FGF receptor
FISH	Fluorescent in situ hybridization
G-CSF	Granulocyte colony-stimulating factor
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial Fibrillary Acidic Protein
GFP	Green fluorescent protein
GBM	Glioblastoma multiforme
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Guanosine monophosphate
GVHD	Graft versus host disease
HCC	Hepatocellular carcinoma
HCT	Hematopoietic cell transplantation
hESCs	Human embryonic stem cells
HPV	Human papilloma virus
HSC	Hematopoietic stem cell
HSCT	HSC transplantation
HSV	Herpes simplex virus
hTERT	Human telomerase reverse transcriptase catalytic subunit
HTP	High-throughput screening
IFN	Interferon
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL	Interleukin
IND	Investigational New Drug
iNOS	Inducible nitric oxide synthase
IP3	Inositol triphosphate
iPSC	Induced pluripotent stem cell
LC	Liquid chromatography
LCM	Laser capture microdissection
LNA	Locked nucleic acid
LNP	Lipid nanoparticle
LOH	Loss of heterozygosity

MAb	Monoclonal antibody
MALDI	Matrix-Assisted Laser Desorption/Ionization
MAP	Mitogen-activated protein
mda	Melanoma differentiation associated gene
MDR	Multidrug resistance
MHC	Major histocompatibility complex
miRNA	microRNA
MMP	Matrix metalloproteinase
MPC	Mesenchymal progenitor cell
MRD	Minimal residual disease
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Mass spectrometry
MSC	Mesenchymal stem cell
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
NASBA	Nucleic acid sequence-based amplification
NCI	National Cancer Institute, USA
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NGS	Next generation sequencing
NHGRI	National Human Genome Research Institute, USA
NIH	National Institutes of Health, USA
NK	Natural killer (cells)
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
NP	Nanoparticle
NSCLC	Non-small cell lung cancer
NT	Neurotrophin
NTF	Neurotrophic factor
ODN	Oligodeoxynucleotide
PBSC	Peripheral blood stem cell
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
PEDF	Pigment epithelium-derived factor
PEG	Poly(ethylene glycol)
PET	Positron emission tomography
PKC	Protein kinase C
PSA	Prostate-specific antigen
PSMA	Prostate-specific membrane antigen
PTEN	Phosphatase and tensin homolog
QD	Quantum dot
rh	Recombinant human
Ras	Retrovirus-associated sequence

RES	Reticuloendothelial system
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcriptase polymerase chain reaction
SAGE	Analysis of gene expression
SC	Stem cell
SCLC	Small cell lung cancer
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SPECT	Single photon emission computed tomography
ssDNA	Single-stranded DNA
SWCNT	Single-wall carbon nanotube
TCR	T cell receptor
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
Tf	Transferrin
TGF	Transforming growth factor
TIL	Tumor-infiltrating lymphocyte
tk	Thymidine kinase
tkRNAi	Transkingdom RNAi
TNBC	Triple negative breast cancer
TNF	Tumor necrosis factor
TOF	Time of Flight
tRNA	Transfer RNA
VEGF	Vascular endothelial growth factor
WGA	Whole genome amplification
WGS	Whole genome sequencing

Chapter 1
Molecular Biology of Cancer

1.1 Introduction

There are multiple factors involved in the causation of cancer, and both external and intrinsic events play a role in the malignant transformation of cells. An understanding of the biochemical abnormalities in tumor cells and the differences from normal cellular biology can lead to the development of effective, nontoxic, tumor-specific treatments.

Cancer is considered to be a genetic disease. The role of genes and oncogenes in cancer is discussed in Chap. 2. Alteration to the DNA inside cells can endow it to grow anywhere and divide indefinitely. Cancer cells differ from their normal counterparts in several ways, some of which are related to their capacity for uncontrolled growth. Analysis often shows increased amounts of DNA per cancer cell, indicating an abnormal increase in chromosomal number, and the nuclei carrying this DNA may take on abnormal shapes. An unusually high percentage of cells in a cancerous growth are undergoing mitosis, as compared with an undetectably low rate of this activity in normal cells. Cancer cells often lose tissue-specific differentiation and indicate a high degree of malignancy. A malignant tumor consists of fully transformed cells that can invade adjacent tissues and spread (metastasize) to other sites in the body to form secondary tumor growths.

1.1.1 The Genesis of Cancer

1.1.1.1 Normal Cell Cycle and Growth

Growth and division of the cell are relevant to the problem of cancer. For the successful completion of the cell cycle, the parental cell must grow large enough to provide each of the daughter cells with complements such as DNA and proteins. Various

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_1,

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steps in the cell cycle also need to be coordinated with the production of cellular material. The cell cycle encompasses two important phases: S phase (DNA synthesis), with duplication of the cellular genome, and M phase (mitotic), with division of the chromosomes and separation of two equal portions as daughter cells. These two phases are separated by two gaps: G1 gap, in which the cell accumulates enzymes required for duplicating the genome, and G2 gap, in which the cell prepares itself for mitosis and makes certain that DNA replication has been successfully completed. Master regulator enzymes trigger the complex events of the cell cycle, and to prevent uncontrolled cell proliferation, each cell is equipped with important guidelines.

Cells can exit from the cell cycle following M phase by entering a rest stop called G0, in which they may remain quiescent for hours, days, or years. Cells can reenter from G0 into active G1 phase in response to various environmental stimuli. Events that damage the regulatory apparatus of the cell may trap it in the active growth cycle and initiate inappropriate growth of a cancer cell. Deregulation of restriction points in the cell cycle appears to be required for tumor development.

Some fully differentiated cells eventually lose their ability to undergo further division and die. In complex organisms, these cells may be replaced by division of primitive stem cells that have a dual potential: to duplicate without differentiation or to proceed toward a fully differentiated, nondividing state. Complex organisms elaborate a large number of extracellular proteins that signal growth and differentiation of stem cells in the hematopoietic lineage. Cancers in the hematopoietic system may arise through overproduction of cells already committed to one pathway or another during blood formation. A cancer may be viewed as a collection of cells whose proliferation and differentiation have become uncoupled and who are not able to complete their program of differentiation.

A single genetic change is rarely sufficient for the development of a malignant tumor. Most evidences point to a multistep process of sequential alterations in several, often many, oncogenes, tumor suppressor genes, or microRNA genes in cancer cells. Mutations of these genes involve elimination of tumor-suppressing proteins and activation of oncoproteins. Methylation of CpG islands located in the promoter regions of a number of tumor suppressor genes has also been considered an important epigenetic step in the process of carcinogenesis. There are other hypotheses as well. One of these implies that breakdown of DNA duplication or repair leads to many thousands of random mutations in cells. Another view points the finger at aneuploidy, the abnormal number of chromosomes in a cell as the first step on the way to cancer.

Tumors often possess cytogenetically different clones that arise from the initial transformed cell through secondary or tertiary genetic alterations. This heterogeneity contributes to differences in clinical behavior and responses to treatment of tumors of the same diagnostic type. Apart from the initial clone and subclones, tumors can also contain progenitor cancer cells, all of which constitute a spectrum of cells with different genetic alterations and states of differentiation. These populations can differ in sensitivity to chemotherapy, radiotherapy, and other treatments, making clinical management difficult. For these reasons, the initiating steps in the development of cancer are of considerable clinical importance and are a priority in the development of rational cancer treatment.

1.1.1.2 Aneuploidy

Cells may become malignant even before any master genes, oncogenes, or tumor suppressor genes are mutated. Carcinogenesis has also been considered as a multistep process in which new parasitic and polymorphic cancer cells evolve from a single, normal diploid cell. A normal cell is converted to a prospective cancer cell either by a carcinogen or spontaneously and evolves spontaneously, over months to decades, to a clinically manifest cancer. The outstanding genotype of such a cell is aneuploidy, an abnormal balance of chromosomes, which increases and varies in proportion with malignancy. Enzymes that normally cooperate to fix DNA begin to fail. Most aneuploid cells die as a result, but a few survive and produce a progeny that are also aneuploid and different from parent cells.

The driving force of carcinogenesis is the inherent instability of aneuploid karyotypes. Aneuploidy renders chromosome structure and segregation error prone, because it unbalances mitosis proteins and the many teams of enzymes that synthesize and maintain chromosomes. The resulting karyotype instability sets off a chain reaction of aneuploidizations, which generate ever more abnormal and eventually cancer-specific combinations and rearrangements of chromosomes. According to this hypothesis, the many abnormal phenotypes of cancer are generated by abnormal dosages of thousands of aneuploid but un-mutated genes.

1.1.1.3 Chromosomal Instability

Chromosomal instability (CIN) can occur early on in the course of cancer genesis. “Master genes,” whose function is critical for a cell to reproduce correctly, may be disabled by mutations or epigenetically, and the cell makes a mistake at every division. The aberrations get worse with each generation. The level of genes in the cell changes as the chromosome pieces are added or deleted. In the course of time, the level of tumor suppressor proteins drops below a critical threshold, whereas extra copies of oncogenes can raise the amount of oncoproteins to dangerous levels, leading to uncontrolled growth of the cells.

No “master genes” have yet been identified, but centrosomes play critical roles in processes that ensure proper segregation of chromosomes and maintain the genetic stability of human cells. They contribute to mitotic spindle organization and regulate cell cycle progression. Centrosomes are abnormal in most aggressive carcinomas, and centrosome defects have been implicated in chromosome instability and loss of cell cycle control in invasive carcinoma. A significant fraction of precursor lesions to some of the most common human cancers had centrosome defects, including in situ carcinomas of the uterine cervix, prostate, and female breast. Moreover, centrosome defects occur together with mitotic spindle defects, chromosome instability, and high cytological grade. Because most preinvasive lesions are not uniformly mutant for p53, the development of centrosome defects does not appear to require abrogation of p53 function. These findings suggest that centrosome defects may contribute to the earliest stages of cancer development through

the generation of chromosome instability. This, together with ongoing structural changes in chromosomes, could accelerate accumulation of alleles carrying pro-oncogenic mutations and loss of alleles containing wild-type tumor suppressor genes and thus accelerate the genomic changes characteristic of carcinoma, the most prevalent human cancer.

CIN is a defining characteristic of most human cancers. Mutation of CIN genes increases the probability that whole chromosomes or large fractions of chromosomes are gained or lost during cell division. The consequence of CIN is an imbalance in the number of chromosomes per cell (aneuploidy) and an enhanced rate of loss of heterozygosity. A major question of cancer genetics is to what extent CIN, or any genetic instability, is an early event and consequently a driving force for tumor progression.

1.1.2 DNA Damage and Repair

In the presence of functional DNA repair pathways, DNA double-strand breaks (DSBs) are mainly repaired by nonhomologous end joining (NHEJ) or homologous recombination (HR), two conserved pathways that protect cells from aberrant chromosomal rearrangements. During the past 2 decades, however, unusual and presumably distinct DNA end-joining repair activities have been unraveled in NHEJ-deficient cells, and these are likely to operate in various chromosomal contexts and species. Most alternative DNA end-joining events reported so far appear to involve microhomologous sequences and are likely to rely on a subset of HR enzymes, namely, those responsible for the single-strand annealing mechanism of HR, and on DNA ligase III. Usually, microhomologies are not initially present at DSB ends and thus need to be unmasked through DNA end resection, a process that can lead to extensive nucleotide loss and is therefore highly mutagenic. In addition to microhomology-mediated end-joining events, recent studies in mammalian cells point toward the existence of a distinct and still ill-defined alternative end-joining pathway that does not appear to rely on preexisting microhomologies and may possibly involve DNA ligase I. Whether dependent on microhomologies or not, alternative DNA end-joining mechanisms are likely to be highly mutagenic in vivo, being able to drive telomere fusion events and cancer-associated chromosomal translocations in mouse models (Decottignies 2013).

1.1.2.1 Mechanism of DNA Damage in Fanconi Anemia Leading to Leukemia

Fanconi anemia is a human cancer predisposition syndrome caused by mutations in 13 FANC genes. The disorder is characterized by genomic instability and cellular hypersensitivity to chemicals that generate DNA interstrand cross-links (ICLs). A central event in the activation of the Fanconi anemia pathway is the

mono-ubiquitylation of the FANCI–FANCD2 complex. Using a cell-free system, it was shown that FANCI–FANCD2 is required for replication-coupled ICL repair in the S phase (Knipscheer et al. 2009). The results of this study show that multiple steps of the essential S-phase ICL repair mechanism fail when the Fanconi anemia pathway is compromised. This finding will not only help shed light on how to fight cancer but how to maintain the stability of the human genome.

1.1.2.2 Accumulation of Random Mutations

As the cell divides, random mutations are introduced and go unrepaired as the genes required to repair the DNA are disabled. As mutations accumulate by the thousands, cancer-related genes are hit. Elimination of tumor suppressor proteins and the activation of oncoproteins short-circuit the autodestruct mechanism of the mutant cell so that it cannot commit suicide and reproduces excessively. After many rounds of mutation and expansion, one cell in the mutants breaks free of all restrictions on its growth. The colony invades adjacent tissues in the host organ.

1.1.2.3 Anticancer Therapy-Related DNA Damage and Repair

Anticancer therapies usually damage tumor cell DNA. DNA damage responses and DNA repair influence the outcome of therapy. However, the relation between DNA repair mechanism efficiency and anticancer therapy might be more complex. Without repair, damage can result in genetic instability and eventually cancer. Because DNA repair normally excises lethal DNA lesions, efficient repair will contribute to intrinsic drug resistance. Paradoxical relationship between DNA mismatch repair and drug sensitivity has been revealed by model studies in cell lines. A publication has reviewed the evidence for the contribution of carcinogenic properties of several drugs as well as of alterations in specific mechanisms involved in drug-induced DNA damage response and repair in the pathogenesis of therapy-related cancers (Casorelli et al. 2012).

1.1.3 Telomeres and Cancer

Specialized nucleoprotein structures, termed telomeres, cap the ends of human chromosomes. These terminal structures, composed of repetitive arrays of guanine-rich hexameric DNA together with specific telomere-binding proteins, play essential roles in protecting the chromosome from damage and degradation. In a noncancerous cell, telomeres are shortened after every round of replication, and when they reach a critically short length, the cell dies. Loss of telomere function is an important mechanism for the chromosome instability commonly found in cancer. Loss of a single telomere can result in ongoing instability, affect multiple chromosomes, and generate many of the types of rearrangements commonly associated with human cancer (Bailey and Murnane 2006).

The presence of telomerase in cancer cells prevents telomere shortening and allows the cells to divide indefinitely. Activation of telomerase, a dedicated reverse transcriptase that synthesizes telomeric sequences, is strongly associated with cancer. Telomeres and telomerase perform important roles in both suppressing and facilitating malignant transformation. Telomerase may be involved in triggering apoptosis, but the underlying molecular mechanism remains unclear.

Telomerase, by virtue of its reverse transcriptase activity and its absence from normal tissues, is considered to be an excellent target for cancer chemotherapy. Treatments that inhibit telomerase activity interfere with the growth of cancer. Several approaches have been developed to inhibit telomerase activity in human cancer cells. Different components and types of inhibitors targeting various regulatory levels have been regarded as useful for telomerase inhibition. Most methods, however, rely on successive telomere shortening. This process is very slow and causes a long time lag between the onset of inhibition and the occurrence of senescence or apoptosis as a reversal of the immortal phenotype. Many telomerase inhibitors seem to be most efficient when combined with conventional chemotherapeutics.

Therapeutic strategies for inhibiting telomerase activity have included both targeting components of telomerase (the protein component, telomerase reverse transcriptase (TERT), or the RNA component, telomerase RNA component (TERC)) or by directly targeting telomere DNA structures. A combination telomerase inhibition therapy has been studied also. The TERT promoter has been used to selectively express cytotoxic gene(s) in cancer cells, and a TERT vaccine for immunization against telomerase has been tested (Siddiqa et al. 2006). Telomerase may have a use in gene therapy. One of the problems in ex vivo gene therapy is the poor proliferation of extracted cells in the laboratory. Insertion of telomerase, alone or in combination with other factors, may enhance the replication capacity so that a larger number of therapeutic cells can be delivered to the patient.

1.1.4 Role of Epigenetics in Cancer

In addition to having genetic causes, cancer is also an epigenetic disease. Epigenetics refers to control of gene expression selectively without affecting the genomic DNA sequence. Epigenetic regulation of gene transcription has emerged as a key biological determinant of cellular differentiation and plays a significant pathogenic role in a number of human diseases, particularly cancer. This regulation is mediated by selective, enzyme-catalyzed, covalent modification of DNA and of proteins (especially histones) that control the conformational transition between transcriptionally active and inactive states of chromatin. Disruption of the activity of disease-associated epigenetic enzymes offers a mechanism-based opportunity for pharmacological intervention in diseases such as cancer. DNA methylation patterns undergo changes in cancer cells and represent an attractive therapeutic target because such epigenetic alterations are more readily reversible than genetic events. When used in combination with conventional chemotherapeutic agents, epigenetic-based therapies may provide a means to sensitize drug-resistant tumors to established treatments.

1.1.4.1 DNA Methylation and Cancer

DNA methylation is an important regulator of gene transcription and plays a role in carcinogenesis. Alterations in DNA methylation are common in a variety of tumors as well as in development. Of all epigenetic modifications, hypermethylation, which represses transcription of the promoter regions of tumor suppressor genes leading to gene silencing, has been most extensively studied. However, global hypomethylation has also been recognized as a cause of oncogenesis. New information concerning the mechanism of methylation and its control has led to the discovery of many regulatory proteins and enzymes. The contribution of dietary folate and methylenetetrahydrofolate reductase polymorphisms to methylation patterns in normal and cancer tissues is under intense investigation. As methylation occurs early and can be detected in body fluids, it may be of potential use in early detection of tumors and for determining the prognosis. Because DNA methylation is reversible, drugs like 5′-azacytidine, decitabine, and histone deacetylase inhibitors are being used to treat a variety of tumors. Novel demethylating agents such as antisense DNA methyltransferase and small interference RNA are being developed, making the field of DNA methylation wider and more exciting.

1.1.5 Metabolic Changes in Cancer

Cancer cells show the Warburg effect, i.e., they consume a large amount of glucose, maintain high rate of glycolysis, and convert a majority of glucose into lactic acid even in the presence of oxygen compared to that of normal cells.

Cancer metabolism is a promising new area of research which deserves further attention. Inhibition of the mammalian target of rapamycin (mTOR) pathway that controls metabolism and proliferation of cells warrants further investigation.

1.1.6 Hallmarks of Cancer

It is difficult to explain why cancer does not affect more persons. Most people make it to old age without getting cancer. A cell must require extraordinary skills to become malignant. The classical six hallmarks of cancer are:

1. Self-sufficiency in proliferative growth signals
2. Insensitivity to growth inhibitory signals from disturbed adjacent tissues that would normally arrest cell division
3. Evasion of apoptosis
4. Induction of angiogenesis
5. Acquisition of a potential for unlimited replication
6. Invasion and metastases

Two emerging hallmarks have been to this list: reprogramming of energy metabolism and evading immune destruction (Hanahan and Weinberg 2011). In addition to cancer cells, tumors show another dimension of complexity as they contain a repertoire of cells that contribute to the acquisition of hallmark traits by creating the "tumor microenvironment." Recognition of the widespread implications of these concepts will influence the development of new methods to treat human cancer. Further complexity has been added to this issue by discovery of cancer stem cells and microRNAs, which will be described later.

1.1.7 Self-Sufficiency of Tumor Proliferation

Normal cells depend upon exogenous growth factors for release from quiescence and for stimulation of DNA synthesis. Cancer cells often counterfeit their own messages to promote growth. Intracellular communication is important for tissue differentiation, and the complex signaling networks are partly mediated by growth factors. There are numerous interactions among growth factors, malignant cells, and microenvironments. Growth factors can influence cell proliferation in a positive or a negative manner. A link has been established between growth factors, receptors with tyrosine kinase activity, and oncogenes. Activation of the growth factor signaling pathway through genetic alterations affecting these genes contributes to the development and progression of cancer. Various growth factors implicated in malignant transformation include the following:

- Epidermal growth factor family
- Fibroblast growth factor family
- Hepatocyte growth factor
- Insulin growth factors
- Neurotrophic factors: prototype example is nerve growth factor
- Platelet-derived growth factor family (includes vascular endothelial growth factors [VEGFs])

Transforming growth factor (TGF)-α seems to promote the entry of cells into normal cycle from a quiescent state and may increase tumor growth by providing an endogenous growth factor mechanism. TGF-β belongs to a family of polypeptide growth factors that inhibit the growth of most epithelial factors. Responsiveness to TGF-β by malignant cells has been linked to differentiation and progression of malignancy. There is evidence for the loss of responsiveness to TGF-β in progression of several cancers such as those involving the breast and the prostate.

1.1.8 Apoptosis

Normal cells, subjected to genetic damage about a critical level, usually activate an autodestruct or suicide program called apoptosis (an ancient Greek word that refers

to falling of leaves off a tree). Apoptosis is a genetically encoded program of cell death characterized by cellular DNA fragmentation, plasma condensation, and membrane blebbing, followed by cell fragmentation and formation of membrane vesicles called apoptotic bodies. Cancer cells can bypass this mechanism. However, the activation of the suicide program is regulated by many different signals that originate from both the intracellular and the extracellular milieu. A class of highly specific proteases, termed caspases, appears to have an important role in apoptosis execution. Mitochondria also appear to have an essential role in apoptosis. The release of apoptogenic protease appears to be under the control of bcl-2 gene product.

Agents of the immune system can sometimes successfully order a cell to self-destruct. One example is cytotoxic T lymphocytes (CTLs) or natural killer cells (NK), which induce apoptosis in targets such as virus-infected cells or tumor cells. In these cases, an effector molecule expressed at the surface of CTLs or NK cells or a soluble cytokine produced by these effector cells is thought to be responsible for target cell death. Fas ligand (FasL), a cell surface molecule belonging to the tumor necrosis family, binds to its receptor Fas and induces apoptosis of Fas-bearing cells. In the immune system, Fas and FasL are involved in downregulation of immune reactions as well as in T-cell-mediated cytotoxicity. Malfunction of the Fas system causes lymphoproliferative disorders and accelerates autoimmune disease.

Inhibitors of apoptosis may be genes normally present in the genome, which become deregulated during oncogenesis. Cancer arises when deranged cells fail to die. Oncogenesis is often associated with defects in p53 or the expression of anti-apoptotic genes, especially bcl-2 family members or oncogenic forms of abl. Similarly, oncogenic Ras has been shown to produce an apoptosis-resistant state.

1.1.8.1 Therapeutic Implications of Apoptosis in Cancer

Apoptosis is rare in cancer because tumor cells have adapted biological pathways to circumvent cell death. Several anticancer therapies aim to induce apoptosis in these cells. Resistance to apoptosis is the principal mechanism whereby tumors are able to develop resistance to cancer therapies. An understanding of how a cell begins the process of apoptosis may help in the design of more targeted cancer therapies.

Cytochrome *c* (CC)-initiated Apaf-1 apoptosome formation represents a key initiating event in apoptosis. These molecules then bind to another protein called Apaf-1 in the cell cytoplasm, and together they form a scaffolding “death wheel” to activate enzymes called caspases that shred a cell apart. But a cell also needs extra energy from ATP to undergo apoptosis, and this extra energy is produced from the “pools” of free nucleotides that exist in the cell cytoplasm. Adding ATP to a cancer cell should impede apoptosis, but a new study has shown that these nucleotide pools, in fact, act not to promote apoptosis through production of ATP but to hinder it (Chandra et al. 2006). They act as “pro-survival factors” that prevent CC, when released from the mitochondria, from “seeing” Apaf-1 in the cytoplasm. The cell

mitochondria needed to release a large and sustained volume of CC to overcome this nucleotide barrier, and they also found evidence that as soon as the release of CC increases, another mechanism kicks in that simultaneously begins to reduce the size of the nucleotide pool to allow CC to bind to Apaf-1. Many cancer drugs focus on pushing the mitochondria to release CC, and not on reducing the nucleotide pool, and the new model suggests that decreasing this pool is essential to produce sensitivity in cancer cells to apoptosis. Cancers such as melanoma and ovarian tumors that quickly become resistant to therapy do so because they have found ways to prevent mitochondria from releasing a lot of CC. Tumor cells also do not want to decrease their nucleotide pool, because they need ATP for continued functioning. An optimal cancer therapy should combine both strategies: maximize release of CC and maximize the decrease of nucleotide levels. Some chemotherapy drugs, like paclitaxel, cisplatin, and etoposide, appear, coincidentally and perhaps inadvertently, to do both and are very effective for specific cancers. However, the new findings provide a theoretical approach that can help in the design of more targeted chemotherapy drugs.

The ratio of apoptosis to mitosis is predictive of tumor growth, with increased apoptosis favoring a positive prognosis. In contrast, necrosis is inversely correlated with apoptosis and predicts a poor prognosis. Results such as these form the foundation for cancer strategies that focus on the regulation of apoptosis. Treatments that restore the ability to properly regulate apoptosis have potential benefits in cancer.

Bcl-2 and apoptosis. The bcl-2 family proteins regulate apoptosis, the natural process by which damaged or unwanted cells die and are cleared from the body. When this process is defective, such as when the bcl-2 family proteins are present in excess, damaged cells can continue to divide, leading to the formation and growth of tumors. The central role that bcl-2 family genes play in the control of programmed cell death and response to chemotherapy suggests that these genes and their encoded proteins provide targets which can be used for improving therapeutic outcomes in patients with advanced cancers.

Effects of biological response modifiers, such as growth factors and cytokines, on the expression of bcl-2 family genes need to be explored with the object of rendering tumor cells more sensitive to induction of apoptosis. Growth of human B-cell lymphomas bearing bcl-2 translocations can be specifically inhibited by antisense oligonucleotides targeted against the bcl-2 gene. One strategy is to overexpress proteins that suppress apoptosis, such as the bcl-2 family protein Mcl-1. The Mcl-1 protein plays a pivotal role in protecting cells from apoptosis and is overexpressed in a variety of human cancers.

Survivin, also called baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), is a protein encoded by the BIRC5 gene. Survivin, a member of the inhibitor of apoptosis (IAP) family, inhibits caspase activation, thereby leading to negative regulation of apoptosis. This has been shown by disruption of survivin induction pathways, leading to increase in apoptosis and decrease in tumor growth. Survivin is a potential target for apoptosis-based therapy in cancer, because cancer cells are targeted while normal cells are spared.

The role of cytokines in apoptosis. Treatment with cytokines and cytotoxic drugs has been shown to sensitize tumor cells to Fas-mediated apoptosis. Fas is a cell surface receptor of tumor necrosis factor (TNF) receptor family. Triggering of Fas by its ligand, FasL, causes apoptosis, provided the cells are sensitized to the Fas signal. For the latter approach, better methods of gene targeting will be needed to replace proapoptotic genes in tumors.

Increase of apoptosis by inhibition of nuclear factor kappa B (NF-κB). The transcription factor NF-κB is activated by chemotherapy and by irradiation in some cancer cell lines and is the principal mechanism of inducible tumor chemoresistance. Inhibition of NF-κB leads to enhanced apoptosis and is a new approach to adjuvant therapy in cancer treatment. This inhibition has been achieved by adenoviral-mediated delivery of a modified form of IκBα, the inhibitor of NF-κB, which sensitizes chemoresistant tumors to the apoptotic potential of TNF-α and chemotherapeutic agent CPT-11, resulting in tumor regression.

Caspase-independent apoptosis. Caspase-mediated apoptosis is a major hindrance to tumor growth and metastasis. Accordingly, defects in signaling pathways leading to the activation of caspases are common in tumors. Moreover, many tumor cells can unexpectedly survive the activation of caspases. As a result, caspase-independent cell death programs are gaining increasing interest among cancer researchers. The heterogeneity of cancer cells with respect to their sensitivity to various death stimuli further emphasizes the need for additional death pathways in the therapeutic control of cell death. An understanding of the molecular control of alternative death pathways is beginning to emerge. Drugs or drug targets engaging caspase-independent death routines already exist, and an example is topoisomerase inhibitor camptothecin, which induces cathepsin-mediated apoptosis-like programmed cell death in hepatocellular carcinoma cells.

1.1.8.2 Autophagy

Although apoptosis is the major mechanism of programmed cell death, cancer therapies may induce modes of cell death different from apoptosis. The identified mechanisms include mitotic catastrophe, autophagy, necrosis, and senescence. Self-cannibalization, called autophagy, occurs when a cell forms a membrane around a part of its cytoplasm or an organelle and then digests the contents, leaving a cavity. A cell that dies from autophagy is riddled with cavities. Cells normally employ autophagy temporarily to survive when nutrients are short, to recycle components to form new organelles, or to fend off viral or bacterial infection. In cancer research, there is evidence both that autophagy is a form of programmed cell death triggered to prevent the replication of damaged cells and that cancer cells in some instances employ it to survive attack. Insights into the molecular mechanisms of autophagy are now leading to the discovery of exciting new potential drug targets.

1.1.9 Tumor Angiogenesis

Angiogenesis (formation of blood vessels) is fundamental to reproduction, development, and repair. During human embryonic growth, vessels develop to deliver adequate nourishment and oxygen from the maternal circulation. Angioblasts of extraembryonic mesoderm give rise to primitive vascular channels, and angiogenesis originates from these structures. After the developing embryo has formed a primary vascular plexus by a process termed vasculogenesis, further blood vessels are generated by both sprouting and non-sprouting angiogenesis, which are progressively pruned and remodeled into a functional adult circulatory system.

Tumor angiogenesis is known for over 100 years, but its mechanism remained obscure. It was identified as an active process induced by humoral tumor-derived stimuli only in the 1960s. In 1971, Judah Folkman proposed the hypothesis that tumor growth is angiogenesis dependent and that endothelial cells may be switched from a resting state to a rapid growth phase by a "diffusible" chemical signal from tumor cells (Folkman 1971). This formed the basis of the concept for antiangiogenesis therapy. Regardless of the mechanism of formation, tumor vessels lose the normal anatomical structural arrangements and can be leaky and fragile, leading to edema and hemorrhage. Functional genomic and proteomic approaches have begun to revolutionize cancer research. The development of powerful technologies, such as DNA microarrays, serial analysis of gene expression, RNA interference, and proteomics, has enabled investigations of gene identification and function at an unprecedented scale. Approaches integrating these technologies with high-throughput forward and reverse genetic screens are already providing insights into the pathomechanism of angiogenesis, leading to the identification of proteins that can be used for selective targeting of tumor vessels (Mittal and Nolan 2007).

Angiogenesis is a complex multistep process involving extensive interplay between cells, soluble factors, and extracellular matrix components. Several angiogenic peptides have been discovered. Some stimulate vascular endothelial cells to proliferate, whereas others act indirectly by mobilizing host cells to release endothelial growth factors. Endogenous inhibitors of angiogenesis (EAIs) counteract the activity of these angiogenic factors. The switch to neoplastic angiogenesis is a tilt in the balance in favor of positive regulators (enhancers) of microvessel growth over the negative regulators (inhibitors).

In a mouse model of pancreatic neuroendocrine cancer, administration of three EAIs—endostatin, thrombospondin-1, and tumstatin peptides—and deletion of their genes reveal neoplastic stage-specific effects on angiogenesis, tumor progression, and survival, correlating with endothelial expression of their receptors. Deletion of tumstatin and thrombospondin-1 in mice lacking the p53 tumor suppressor gene leads to increased incidence and reduced latency of angiogenic lymphomas associated with diminished overall survival (Xie et al. 2011). The results demonstrate that EAIs are part of a balance mechanism regulating tumor angiogenesis, serving as intrinsic microenvironmental barriers to tumorigenesis.

Galectin-1 (gal-1) is a receptor for the angiogenesis inhibitor anginex, and this protein is crucial for tumor angiogenesis. Gal-1 is overexpressed in endothelial cells of different human tumors. Expression knockdown in cultured endothelial cells inhibits cell proliferation and migration. The importance of gal-1 in angiogenesis is illustrated in the zebrafish model, where expression knockdown results in impaired vascular guidance and growth of dysfunctional vessels. The role of gal-1 in tumor angiogenesis is demonstrated in gal-1-null mice, in which tumor growth is markedly impaired because of insufficient tumor angiogenesis (Thijssen et al. 2006). Furthermore, tumor growth in gal-1-null mice no longer responds to antiangiogenesis treatment by anginex. Thus, gal-1 regulates tumor angiogenesis and is a target for angiostatic cancer therapy.

The well-documented role of VEGF in tumor angiogenesis has led it to become one of the leading therapeutic targets for the treatment of cancer. Emerging evidence from genetically modified animal models, however, suggests that elevated levels of VEGF may inhibit, rather than promote, early tumor development and progression. For example, hypermorph VEGF transgenic mice display delayed progression of a retroviral-induced murine leukemia, and knockdown of VEGF expression within the myeloid compartment accelerates tumor progression (Vecchiarelli-Federico et al. 2010). Several mechanisms have been proposed to explain this paradox, whereby VEGF induces changes within the hematopoietic compartment and tumor microenvironment through recruitment of tumor inhibitory monocytic cells and the negative regulation of tumor angiogenesis. Therefore, the levels of VEGF expression in both tumor and nontumor tissues, as well as the context and timing of its modulation relative to cancer induction, play an important role in determining the effects of VEGF expression on tumorigenicity.

1.1.10 Acquisition of a Potential for Unlimited Replication

Changes in cancer genes endow a cell with powers that allow it to outbreed its neighbors. The cell passes its abnormalities in the DNA sequence to its descendants so that the clone army keeps on growing and advancing.

1.1.11 Invasion and Metastases

Cancers usually become life threatening only after they disable the cellular circuitry that confine them to a specific part of the particular organ in which they arose. Genetic alterations in tumors occur in varying orders; many of them concomitantly influence invasion as well as the other cancer-related cellular activities, genes encoding elements of the cadherin/catenin complex, the nonreceptor tyrosine kinase Src, the receptor tyrosine kinases c-Met and FGF receptor (FGFR), the small GTPase Ras, and the dual phosphatase and tensin homolog (PTEN) are among those involved.

There are numerous clinical and experimental observations showing that invasion results from the cross-talk between cancer cells and host cells, comprising myofibroblasts, endothelial cells, and leukocytes, all of which are themselves invasive. In bone metastases, host osteoclasts serve as targets for therapy. The molecular analysis of invasion-associated cellular activities, i.e., homotypic and heterotypic cell–cell adhesion, cell–matrix interactions, and ectopic survival, migration, and proteolysis, reveal branching signal transduction pathways with extensive networks between individual pathways. Cellular responses to invasion-stimulatory molecules such as scatter factor, chemokines, leptin, trefoil factors, and bile acids or inhibitory factors such as platelet-activating factor and thrombin depend on activation of trimeric G proteins, phosphoinositide 3-kinase, and the Rac and Rho family of small GTPases. The role of proteolysis in invasion is not limited to breakdown of extracellular matrix but also causes cleavage of proinvasive fragments from cell surface glycoproteins.

Metastasis or spread of tumors to sites in the body beyond the primary location is the most malignant manifestation. To possess metastatic potential, a cell has to be able to penetrate the epithelial basement membrane and invade the surrounding tissue. Then the tumor cells spread via the lymphatics or the bloodstream or both, extravasate, and multiply at secondary sites. Growth of tumors beyond 1 cm^3 requires vascularization of solid tumors. Angiogenesis not only allows further growth of tumors but may facilitate hematogenous spread as well. Metastases present the greatest obstacle to successful cancer therapy. Despite all the advances made in conventional cancer treatments and surgical techniques, most of the cancer deaths result from metastases. An understanding of the molecular mechanisms underlying metastases is leading to novel cancer therapies which might provide a means for disruption of metastasis formation.

Although tumorigenesis is necessary for formation of metastases, uncontrolled growth alone will not produce metastatic phenotype. This multistep process requires additional genetic changes, positive modulators (oncogenes), and loss of negative regulators (tumor growth suppressors). Thus, tumorigenesis and metastasis have both overlapping and separate features.

Most common organs involved by metastases are the lungs, the brain, and the bones. It is unclear as to why particular cancers preferentially metastasize to certain sites. The possibilities include differential survival and proliferation at these sites, or selective trapping with or without preferential homing as in the case of breast cancer. Chemokine receptors CXCR4 and CCR7 are found on breast cancer cells and their ligands are highly expressed at sites associated with breast cancer metastases. This results in chemotaxis, or directed migration of tumor cells from their primary site via the circulation to the preferential sites of metastases.

1.2 Ion Channels and Cancer

Ion channels are protein pores in the cell membrane that allow the passage of ions down their respective electrochemical gradients. Ion channels are classified according to the ion passing through them (e.g., sodium, potassium, calcium, or chloride)

Table 1.1 Ion channels involved in development of cancer

Ion channel	Effect on cancer cell lines	Mechanism
VGKCs	Tumor progression and invasion	Cell proliferation
VGSCs	Metastatic carcinoma	Cell motility
VGCCs	Prostate cancer	Cell differentiation
	Small-cell lung cancer	Cell motility
TRPM1	Melanoma	Cell growth
TRPM7	Glioma	Cell proliferation
TRPM8	Prostate, breast, lung, and skin cancers	Proliferation and apoptosis
TRPV	Colorectal and prostate carcinoma	Cell proliferation

Based on data from Li and Xiong (2011)

and the mechanisms by which they are opened or closed. There are two basic types of ion channels: (1) voltage-gated ion channels (VGICs) and (2) ligand- or transmitter-gated ion channels; but some channels exhibit dual gating mechanisms. VGICs may be voltage-gated sodium channels (VGSCs), voltage-gated calcium channels (VGCCs), or voltage-gated potassium channels (VGKCs). Both voltage-gated and ligand-gated ion channels play important roles in physiological conditions such as electrical signaling, gene expression, hormone secretion, learning, and memory. However, in pathological conditions, the property or activity of ion channels may change and may promote the growth and proliferation of tumor cells.

Transient receptor potential (TRP) channels, transmembrane cation-permeable channels that regulate intracellular Ca^{2+} and Na^{+} concentrations as well as membrane voltage, are widely expressed in mammalian tissues. TRPM subfamily, also called melastatin-like TRP channels, consists of eight members: TRPM1/3, TRPM4/5, TRPM6/7, TRPM2, and TRPM8. Ion channels represent promising targets for developing novel and effective cancer therapies (Li and Xiong 2011). The role of ion channels in drug discovery for cancer is described in Chap. 14. Ion channels involved in cellular processes vital to the progression and development of cancer are shown in Table 1.1.

1.3 Cancer Microenvironment

All cancers contain a diverse population of cells, including cancer cells (typically refer to those cells harboring genetic mutations), stromal fibroblasts, endothelial cells, inflammatory cells, and abundant extracellular matrix. The cancer microenvironment, composed of noncancer cells and their stroma, has become recognized as a major factor influencing the growth of cancer. The microenvironment has been implicated in the regulation of cell growth, determining metastatic potential and possibly determining location of metastatic disease and impacting the outcome of therapy. While the stromal cells are not malignant per se, their role in supporting cancer growth is so vital to the survival of the tumor that they have become an attractive target for chemotherapeutic agents. Various cellular and molecular

components of the stromal environment, their effects on cancer cell dynamics, and the rationale and implications of targeting this environment for control of cancer have been reviewed elsewhere (Li et al. 2007).

Despite the importance of tumor–stromal interactions, there is a limited understanding of the complex relationship between the tumor cells and the surrounding stromal cells. It is now acknowledged that tumor cells and their stroma coevolve during tumor development and progression. However, how stromal cells are altered during tumor progression and how they reciprocally influence tumor initiation and progression are poorly understood. Tumor microenvironments are attractive targets for therapy, because they are presumably genetically stable, which is in contrast to tumor cells that are known to be genetically unstable and thus can accumulate adaptive mutations and rapidly acquire drug resistance. Manipulating tumor–stromal interactions has the potential to revert the malignant phenotype, which may eventually lead to reduction or elimination of metastasis-associated morbidity and turn metastatic cancer into a manageable chronic disease.

A study indicates that hypoxia leaves a specific mark on miRNA profiles in a variety of cell types, with a critical contribution of the hypoxia-inducible factor, and a subgroup of these hypoxia-regulated miRNAs seem to play a role in cell survival in a low-oxygen environment (Kulshreshtha et al. 2007). Based on comparison of hypoxia-associated miRNA spectra with published data from a large number of tumors, it is proposed that cancer-associated miRNA profiles exhibit a hypoxic signature.

1.4 Epidemiology of Cancer

Despite numerous advances in medicine, cancer remains a major health problem. Approximately 13 million new cases of cancer are diagnosed worldwide annually, of which about half die. More than 11 million Americans are living with cancer or the prospect that cancer may return. Approximately 1.6 million new cases of cancer were reported in the USA in 2012, and cancer burden might increase with aging US population. At current incidence rates, the total number of new cancer cases is expected to nearly double by 2050 to three million. Cancer remains the second biggest cause of death in the USA, after heart disease, and nearly one in four Americans is projected to die from cancer. There is more hope for the future, as better drugs and better methods of delivery are developed. Statistics on selected cancers in the USA for 2012 are shown in Table 1.2.

1.5 Current Management of Cancer

For many years, standard cancer therapy has been based on surgery, radiotherapy, and chemotherapy, used alone or in combination. It is recognized that advanced cancers cannot be cured with single drugs. They often are resistant to single agents,

Table 1.2 Estimated new cases of cancer in the USA in most involved organs (2012)

Males: number of cases = 810,040		Females: number of cases = 762,870	
Organ involved	% of total	Organ involved	% of total
Prostate	33	Breast	32
Lung and bronchus	13	Lung and bronchus	12
Colon and rectum	10	Colon and rectum	11
Urinary bladder	7	Uterine corpus	6
Melanoma of skin	5	Melanoma of skin	4
Non-Hodgkin's lymphoma	4	Non-Hodgkin's lymphoma	4
Kidney	3	Ovary	3
Leukemia	3	Thyroid	3
Oral cavity	3	Urinary bladder	2
Pancreas	2	Pancreas	2
All other sites	17	All other sites	21

Source: American Cancer Society

and even if they are initially sensitive, their molecular heterogeneity usually guarantees the secondary outgrowth of rare cells that are resistant. In contrast, drug combinations can cure some types of cancers even at advanced stages. Examples of effective therapeutic combinations include doxorubicin, bleomycin, vinblastine, and dacarbazine for Hodgkin's lymphoma and bleomycin, etoposide, and cisplatin for testicular cancer.

Surgery can effectively remove some tumors, but surgery alone cannot effectively treat cancers that have spread widely through the body. Radiotherapy and chemotherapy can kill cancer cells and shrink tumors, but at safe doses, they may fail to eradicate all cancer cells while at the same time causing side effects by damaging healthy cells.

1.5.1 Chemotherapy

Currently available anticancer drugs can be divided into three broad categories:

1. Those that interact with DNA (cytotoxics)
2. Those that associate with membrane receptors (hormonal agents)
3. Those that bind to antigen expressed at the cell surface

The principal mechanism of action of each of the cytotoxic drugs is uncertain, although all of them produce DNA damage. Alkylating agents induce arrest of DNA transcription/replication. Antimetabolites induce DNA injury by inhibiting thymidine synthetase, purine synthesis, or DNA repair. The anthracyclines damage DNA by intercalating with DNA, generating free radicals, or interacting with DNA-modifying enzyme topoisomerase. The final common pathway of cytotoxic-induced cell death is via apoptosis.

1.5.1.1 Limitations of Cancer Chemotherapy

Despite the introduction of numerous chemotherapeutic agents, cancer survival rates for clinically advanced cancers have improved little during the past quarter of a century. Although notable successes have been achieved in the treatment of acute leukemias and malignant lymphomas, advanced solid malignancies (with the exception of testicular cancer and some other rare neoplasms) are usually incurable. The major limitations of chemotherapy are toxicity and multidrug resistance.

All of the commonly used chemotherapy agents are associated with acute toxic effects. These effects are a limiting factor in the dosing levels of these substances. Various supplementary treatments are used to counteract the adverse effects. For example, myelotoxicity is treated with colony growth-stimulating factors such as G-CSF, while other treatments have been developed to counteract nausea and vomiting. One major adverse effect of chemotherapy in long-term survivors is the development of secondary malignancies as a result of alkylating agents. Various measures to reduce the incidence of chemotherapy-associated toxicity include combination therapies, development of less toxic chemotherapeutic agents, and improved targeted methods of drug delivery to avoid systemic toxicity.

Several chemotherapy regimens include combinations of cytotoxic agents, each of which has been shown to be effective as a single agent against the cancer type to be treated. The toxicity profiles of the individual drugs are not superimposed to limit the severity of toxicity. Dosage schedules are chosen to optimize tumor response and toxicity profile. Scheduling of myelosuppressive chemotherapy is limited by bone marrow recovery. Regimens for a myelosuppressive therapy alternating with non-myelosuppressive therapy are used in such cases. High-dose chemotherapy has been combined with autologous bone marrow or peripheral stem cell rescue as a salvage therapy.

1.5.2 Radiotherapy

Radiotherapy is an established treatment for certain types of cancer. The role of radiotherapy for a particular tumor is determined by the radiosensitivity of the tumor. Pharmacological agents can interact with radiotherapy in several ways, and attempts are being made to determine how drugs and radiation can be combined in an optimal manner. Radiotherapy has been claimed to have a curative role as a sole treatment for some cancer, as well as being a component of multimodal treatments (including chemotherapy) for breast cancer and soft tissue sarcoma. For cancers with both local control problems and metastatic potential, experts generally agree that a combination of chemotherapy and radiotherapy is more effective than either approach alone.

1.5.2.1 Brachytherapy

Brachytherapy, or short-range radiation therapy, involves implanting small, rice-sized radioactive pellets ("seeds") directly into the tumors to confine and target treatment within the cancer site. The implants deliver radiation to the cancer, gradually over time.

1.5.3 Surgery

Surgery plays an important role in the multidisciplinary management of cancer although it fails to cure cancer in more than half the patients. In the absence of metastases, the surgeon can ablate the primary tumor with a reasonable chance of cure. This depends on the location of the tumor and the completeness of the excision. In the case of glioblastoma multiforme of the brain, it is not possible to remove the tumor completely. Surgical methods are combined with other modalities such as chemotherapy and radiotherapy as well as innovative methods of cancer drug delivery during surgery. Surgical procedures may be involved in implantation of anticancer therapeutic substances.

1.6 Role of "Omics" in Oncology

There are >100 technologies ending with the suffix "omics" that are relevant to cancer. The most important of these are genomics (see Chap. 2), proteomics (see Chap. 4), transcriptomics, and metabolomics. Transcriptomics is the study of the entire set of RNA transcripts of an organism. Metabolomics is the study of the small molecules, or metabolites, contained in a human cell, tissue, or organ (including fluids) and involved in primary and intermediary metabolism. A related term metabonomics is the use of nuclear magnetic resonance (NMR) technology to study metabolomics.

Systems biology (see Chap. 16) requires the use of a variety of analytical platforms including "omics" as well as bioinformatics, data integration, and modeling. This concept fits in with the concept of personalized medicine by integrating several technologies.

1.7 Historical Landmarks in Cancer Detection and Treatment

Historical landmarks in drug delivery for cancer are shown in Table 1.3.

Table 1.3 Historical landmarks in cancer detection and treatment

Year	Landmark
3000 BC	The first description of a disease resembling cancer in Egypt: Edwin Smith Papyrus
460–370 BC	Hippocrates used the term carcinos (crab in Greek) to describe cancer. Celsus (28–50 BC) translated the term into cancer (Latin), and Galen (130–200 AD) used "oncos" for swelling
1847	The first laboratory test for a protein cancer biomarker, the Bence Jones protein in urine
1895	Discovery of X-rays followed shortly by introduction of radiotherapy as treatment for cancer
1898	Discovery of radium by Marie Curie
End of nineteenth century	Paul Ehrlich envisioned antibodies as magic bullets that would specifically tract and kill microorganisms and cancer (Schwartz 2004)
1906	First concept of drug targeting to a specific site in the body by Paul Ehrlich
1932	Discovery of neutrons which later became the basis of boron neutron capture therapy (Laramore 1997)
1940s	Origin of nitrogen mustards as first anticancer therapy targeting all tumor cells (Papas 2001)
1957	First hematopoietic stem cell transplantation: radiation and chemotherapy for cancer followed by the intravenous infusion of bone marrow (Thomas et al. 1957)
1958	First human bone marrow transplant as treatment for leukemia (Mathe and Schwarzenberg 1979)
1958	Introduction of 5-fluorouracil, chemotherapy for cancer
1958	Introduction of brachytherapy: implanting small radioactive pellets directly into the tumors
1965	Discovery of liposomes (Bangham et al. 1965), used later as vehicles for anticancer drug delivery
1971	Report of carcinoembryonic antigen (CEA) as biomarker of cancer (Moore et al. 1971)
1971	Introduction of the concept that tumor growth is angiogenesis dependent (Folkman 1971)
1973	Discovery of "dendritic cells," which elicited vivid T-cell response to foreign substances, indicating their importance in the adaptive immune response (Steinman and Cohn 1973). It provides new avenues for prevention and therapy against cancer
1975	Generation of murine monoclonal antibodies that were later used for targeted delivery of anticancer therapies (Kohler and Milstein 1992)
1985	Discovery of polymerase chain reaction (Mullis et al. 1986)
1987	Cancer targeting with nanoparticles coated with monoclonal antibodies (Douglas et al. 1987)
1989	First experiment on gene therapy of patients with advanced cancer using tumor-infiltrating lymphocytes transduced with genes coding tumor necrosis factor (TNF) (Rosenberg et al. 1990)
1992	First use of antisense oligonucleotide in patients: for treatment of leukemia (Bayever et al. 1992)
1995	FDA-approved Doxil, a liposomal intravenous formulation of doxorubicin, for the treatment of Kaposi's sarcoma. Drug carried by nanosized liposomes is less toxic with targeted delivery

(continued)

Table 1.3 (continued)

Year	Landmark
1997	Approval by the FDA of rituximab, the first therapeutic antibody for non-Hodgkin's lymphoma
2001	Sequencing of the human genome completed opening the way for study of cancer genes
2001	RNA interference shown to have anticancer effect
2001	FDA approved the first precision targeted anticancer drug, imatinib (Gleevec), to treat chronic myeloid leukemia and gastrointestinal stromal tumor
2003	Approval in China of the first product for gene therapy of cancer
2005	FDA-approved Abraxane™, a taxane based on nanotechnology, for the treatment of breast cancer. Nanoparticle form of the drug overcomes insolubility problems encountered with paclitaxel and avoids the use of toxic solvents
2008	Sequencing of the first whole cancer genome in a patient with acute myeloid leukemia (Ley et al. 2008)

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Chapter 2
Oncogenomics

2.1 Introduction

It is important to realize that cancer is not just one but many different diseases, each with distinct characteristics and therapeutic requirements. Genomic research has provided a stratification of cancer types. Genomics as applied to cancer or oncogenomics provides an unprecedented opportunity for the discovery of potential new targets. Through the establishment of a suite of research programs, the National Cancer Institute (NCI) of the USA is developing the interface of genomics and cancer. The components of the program, including the Cancer Genome Anatomy Project (CGAP), the Cancer Molecular Analysis Project, and the Initiative in Chemical Genetics, provide a platform for the integration of basic and clinical research for the benefit of patients' health. Oncogenomics has assumed importance as a guide to management and development of personalized cancer therapies. Gene expression profiling of tumors is also used for developing targeted anticancer therapies.

2.2 Cancer Genes

Progression of a single cell from a normal to a neoplastic state always involves a series of genetic changes that alter either the regulation or the function of a variety of different genes. Such genes may play roles in a number of overlapping physiological processes, including genome maintenance, cell cycle control, apoptosis, contact inhibition, invasion and metastasis, and angiogenesis. These cancer genes are often classified in two main categories: oncogenes and tumor suppressor genes. The distinction between these two categories is that tumor progression is promoted by overexpression or gain of function in oncogenes but by nonexpression or loss of function in tumor suppressor genes. Most highly penetrant cancer predispositions are thought to be caused by germline mutations in tumor suppressor genes, but the

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_2,

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same phenomenon can occur with germline mutations in oncogenes. For example, rare germline mutations in the RET tyrosine kinase predispose to endocrine neoplasms.

2.2.1 Oncogenes

Oncogenes are genes associated with neoplastic proliferation following a mutation or perturbation in their expression. Oncogenes are part of the signal transduction pathway, and various examples include growth hormones, receptors, G proteins, protein kinases, transcription factors, and cyclins. Our understanding of oncogenes originated with the discovery of certain viral genetic elements that are responsible for the tumor-forming ability of retroviruses. Retroviruses have three genes: eng and gag coding for structural proteins and pol coding for reverse transcriptase (the enzyme that enables RNA to be converted into complementary DNA [cDNA]). A fourth gene gives retroviruses the ability to induce tumor growth in vivo. An example of this is the tax gene in case of the HTLV (human T-cell leukemia virus).

The antecedent genes or proto-oncogenes play an essential physiological role in normal cellular proliferation and differentiation. Proto-oncogenes do not have the transforming potential to form neoplasms in their native state. They can induce cancer, however, when they are captured and subverted by retroviruses (RNA viruses). In general, they appear to act on biochemical pathways by which growth factors stimulate cellular proliferation. Most hematopoietic tumors and soft tissue sarcomas are initiated by the activation of an oncogene, followed by alterations in tumor suppressor genes and other oncogenes. In contrast, most carcinomas are initiated by the loss of function of a tumor suppressor gene, followed by alterations in oncogenes and additional tumor suppressor genes.

2.2.2 Tumor Suppressor Genes

Cellular genes that regulate cell growth by counteracting the action of proto-oncogenes are called “anti-oncogenes” or tumor suppressor genes. Potential sites where these genes might inhibit the development of cancer include cell proliferation, differentiation, and senescence; cell-to-cell communication; and chromosomal stability. Mutations in tumor-suppressing genes cause growth-inhibiting proteins encoded by the genes to disappear, allowing the cell to survive and continue dividing when normally it should not. An excess of oncoproteins and lack of tumor suppressor proteins lead mutant cells to reproduce excessively.

In general, both alleles of tumor suppressor genes must be disrupted to observe a phenotypic effect. Broadly speaking, there are two types of tumor suppressor gene: “gatekeepers” and “caretakers.” In contrast to gatekeepers, caretaker genes do not directly regulate proliferation, but act to prevent genomic instability.

Thus, mutation of caretaker genes leads to accelerated conversion of a normal cell to a neoplastic cell. Many caretaker genes are required for the maintenance of genome integrity and play a role, directly or indirectly, in the repair of DNA strand breaks by the homologous recombination pathway, failure of which is associated with chromosome instability and cancer-prone clinical syndromes. This mechanism might provide the possibility of cancer prevention approach such as stimulation of alternative repair pathways or new treatment approaches to exploit the repair deficiency in tumors.

The list of tumors associated with homozygous loss of specific chromosomal loci is growing rapidly. In addition, in vitro evidence supports the existence of tumor-suppressing genes (Table 2.1). To create these genes, fusion of a normal cell with a malignant cell produces a hybrid in which the tumorigenic phenotype is usually suppressed; the differentiation program of the normal parent cell may then be imposed upon this hybrid.

2.2.2.1 p53

The p53 gene (named for its 53-kDa mass) is the best known of tumor suppressor genes. This gene encodes a nuclear protein that binds to and modulates the expression of genes important for DNA repair, cell division, and cell death by apoptosis. Apoptosis has been linked to p53 function in some cell types; by the same token, loss of p53 function renders cells resistant to apoptosis in many cases. Mutations of the p53 gene are the single most common genetic alteration observed in human cancers; a mutant p53 gene has been detected in nearly half of human cancers. The p53 gene is located on chromosome 17p13.1, which is one of the more frequent targets for chromosome alterations in human cancer. Although this gene is not required for normal development, lack of p53 function raises the risk of cancer substantially.

Chemical changes to DNA packaging proteins called histones regulate transcription or activation of p53 and other target genes, which has major implications for the treatment of cancer. Several proteins may be required for gene activation by the tumor suppressor p53 in a single early step in this process. Various cofactor functions, as well as underlying mechanisms are involved in distinct histone modifications in p53-dependent gene activation.

2.2.2.2 p16

The normal function of the protein encoded by the tumor suppressor gene p16 is to inhibit function of the cyclin D–CDK complexes. Thus, loss of p16 function, which occurs in roughly 50 % of all human tumors, has a phenotype similar to overexpression of the proto-oncogene cyclin D1, i.e., it promotes cell cycle progression from the G1 to the S phase.

Table 2.1 Tumor suppressor genes, their chromosomal location, function, and associated tumors

Gene	Chromosomal location	Function	Associated tumor
APC	5q21	β-Catenin binding, communicates between cell surface proteins and microtubules	Familial adenomatous polyposis coli
BRCA1	17q21-22	Tumor suppressor gene (unknown function)	Inherited susceptibility to breast and ovarian cancer
BRCA2	13q12-13	Tumor suppressor gene (unknown function)	Hereditary breast cancer
CDK4	12q13	Cyclin-dependent kinase	Hereditary melanoma 2
p16 (CDK2A)	9p21	p16-cyclin-dependent kinase inhibitor	Germline mutations cause hereditary melanoma
DCC	18q21	Cell adhesion	Colorectal cancer
EXT1	8q24.1	Tumor suppressor gene (unknown function)	Langer–Giedion syndrome
FHIT	3p24.3	Tumor suppressor gene altered by exposure to environmental agents	50 % of gastrointestinal cancers
MSH2	2p16	Mismatch repair genes	Hereditary nonpolyposis colorectal cancer
MLH1	3p21		
PMS2	7p22		
NF1	17q11.2	GTPase-activating protein (GAP) for *ras* from neural crest-derived cells	von Recklinghausen's neurofibromatosis
NF2	22q11.1	Integration of cytoskeleton with plasma membrane	Acoustic neuroma, bilateral meningiomas
P53	17p13	Transcription factor, regulates cell cycle and apoptosis	Germline mutations cause Li–Fraumeni syndrome
PTC	9q22.3	Membrane protein involved in hedgehog protein signal transduction	Basal-cell carcinoma
PTEN	10q23.3	PTEN (phosphatase and tensin homolog), a tumor suppressor gene, prevents cells from dividing rapidly	Prostate cancer, Cowden syndrome (genetic disorder with high risk of developing cancer)
RB1	13q14	Regulates transcription factors (E2F-DP1), regulates cell cycle	Retinoblastoma
RET	10q11	Receptor tyrosine kinase	Medullary thyroid cancer. Multiple endocrine neoplasia 2
TSC2	16p13	Tumor suppressor gene (unknown function)	Tuberosclerosis 2
VHL	3p25	Elongin (transcription elongation)	von Hippel–Lindau syndrome
WT1	11p13	Zinc finger transcription factor	Wilms' tumor, nephroblastoma

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Whereas somatic mutations are responsible for most p53 and p16 lesions in human tumors, rare germline mutations of p53 cause Li–Fraumeni syndrome, and germline mutations of p16 cause predisposition to melanoma. Germline mutations of tumor suppressor genes manifest their effects in an autosomal-dominant fashion, because each cell in a mutation carrier starts with only one normal copy of the tumor suppressor gene. Within individual cells, somatic mutation of the remaining normal allele occurs frequently enough that the carrier experiences a markedly elevated lifetime cancer risk.

2.2.2.3 Rb Gene

Rb, a tumor suppressor gene, is linked to two key processes that frequently malfunction when cancer begins—proliferation and apoptosis. The Rb tumor suppressor pathway is functionally inactivated in most human cancers. Loss of Rb expression has been widely reported in both acute myeloblastic and acute lymphoblastic leukemias. Mutations in Rb are linked to several types of cancer including the childhood disease retinoblastoma.

2.2.2.4 BRIT1 Gene

A signaling network of molecular checkpoint pathways protects the human genome by detecting DNA damage, initiating repair, and halting division of the damaged cell so that it does not replicate. The gene, BRIT1, activates two of these checkpoint pathways: the ATM pathway springs into action in response to damage caused by ionizing radiation and the ATR pathway responds to DNA damage caused by ultraviolet radiation. BRIT1 is underexpressed in human ovarian, breast, and prostate cancer cell lines, and defects in BRIT1 seem to be a key pathological alteration in cancer initiation and progression (Rai et al. 2006). Disruption of BRIT1 function abolishes DNA damage responses and leads to genomic instability, which fuels the initiation, growth, and spread of cancer. By using siRNA to silence the BRIT1 gene, the scientists shut down both checkpoint pathways in cells exposed to either type of radiation. siRNA was then used to silence the gene in normal human mammary epithelial cells (HMECs). With the result that inactivation of the gene caused chromosomal aberrations in 25 % of cells. Control group HMEC had no cells with chromosomal aberrations. In cells with the gene silenced that were then exposed to ionizing radiation, 80 % of cells had chromosomal aberrations. Reduced BRIT1 expression is also found in advanced epithelial ovarian cancer as well as in prostate cancer tissue compared with noncancerous cells. Thus, BRIT1 may function as a tumor suppressor, and as such, further understanding of its function may well contribute to novel, effective therapeutic approaches for cancer.

2.2.2.5 Tumor Suppressor Genes and Metastases

Although search for a specific metastatic-inducing gene continues, transfection with oncogenes has been shown to induce metastatic potential in tumor cell models. Tumor suppressor genes can encode proteins that partly inhibit the metastatic cascade. Loss of expression of inhibitory proteins may lead to progression of a tumor to a metastatic state.

FoxM1 transcription factor gene, stimulated by oncogenic signaling pathways and reactive oxygen species, is overexpressed in cancer. This depends upon activation by cyclin and cyclin-dependent kinases as well as Plk1. FoxM1 stimulates expression of several genes involved in the cell cycle progression and supports proliferation of tumor cells by stimulating expression of the antioxidant genes and reducing oxidative stress. A study provides evidence that FoxM1, in the absence of its inhibitor, the tumor suppressor Arf, drives metastasis of hepatocellular carcinoma (Raychaudhuri and Park 2011). It induces an epithelial–mesenchymal-like transition phenotype in cancer cells, increases cell migration, and induces premetastatic niche at the distal organ of metastasis. FoxM1 directly activates genes involved in multiple steps of metastasis.

2.2.3 Role of Bub 1 Gene in Cell Division

Bub 1 is an enzyme that controls several processes required for cell division to occur (Perera et al. 2007). It is a component of the spindle assembly checkpoint, a surveillance mechanism that ensures genome stability by delaying anaphase until all the chromosomes are stably attached to spindle microtubules. Discovery of the gene that generates Bub 1 is significant because if it is switched off, then the cells are unable to divide successfully and die. Mouse embryos lacking the Bub 1 gene are unable to develop. Bub 1 inactivation in adult males inhibits proliferation in seminiferous tubules, reducing sperm production and causing infertility. Unlike some other genes that become mutated in cancer cells, the Bub 1 gene appears normal indicating that it behaves in exactly the same way in cancer cells as it does in healthy cells. Because Bub 1 had such a profound effect on cell division at all stages of a cell's life, it is hoped that will have a similar effect on cancer cells and stop proliferation of the tumor. This may be a new way of destroying tumors and could lead to alternative methods of cancer treatments. Drugs are already being developed that are able to block the actions of Bub 1-type enzymes, known as "protein kinases"; such kinase blockers or "inhibitors" are already providing a whole new approach to tackling cancer, and Bub 1 inhibitors may be another weapon in the oncologist's arsenal.

2.2.4 Anticancer Treatments Based on RNA Regulation of Genes

RNA plays an important and direct role in the synthesis of proteins, but not all types of RNA are directly involved in this activity. One particular type of RNA is involved in regulation of the dihydrofolate reductase gene (DHFR), determining whether the gene is “on” or “off” (Martianov et al. 2007). In quiescent cells the mechanism of transcriptional repression of the major promoter of the gene encoding DHFR depends on a noncoding transcript initiated from the upstream minor promoter and involves both the direct interaction of the RNA and promoter-specific interference. The specificity and efficiency of repression is ensured by the formation of a stable complex between noncoding RNA and the major promoter, direct interaction of the noncoding RNA with the general transcription factor IIB, and dissociation of the preinitiation complex from the major promoter. By using in vivo and in vitro assays such as inducible and reconstituted transcription, RNA bandshifts, RNA interference, chromatin immunoprecipitation, and RNA immunoprecipitation, it was shown that the regulatory transcript produced from the minor promoter has a critical function in an epigenetic mechanism of promoter-specific transcriptional repression.

The DHFR gene produces an enzyme that controls thymine production, necessary in rapidly dividing cells, and thus plays a key role in control of tumor growth. Inhibiting the DHFR gene could help prevent the growth of ordinary cells which develop into cancer. The first anticancer drug, methotrexate, acts by binding and inhibiting the enzyme produced by this gene. Understanding how we can use the RNA to switch off or inhibit DHFR and other genes may have important therapeutic implications for developing new anticancer treatments.

2.2.5 Interaction of Cancer Genes

How driver mutations in cancer genes interact to promote tumor development is not well understood. Renal cell carcinoma (RCC) provides an opportunity to study complex relationships among cancer genes. The four most commonly mutated genes in RCC of clear-cell type (the most common type) are two-hit tumor suppressor genes, and they cluster in a 43-Mb region on chromosome 3p that is deleted in ~90 % of tumors (Peña-Llopis et al. 2013). Meta-analyses conducted by these authors show that mutations in PBRM1 and SETD2 co-occur in tumors at a frequency higher than expected by chance alone, indicating that these mutations may act together in tumorigenesis. In contrast, consistent with their previous results, mutations in PBRM1 and BAP1 tend to be mutually exclusive, which may indicate negative genetic interactions. Mutations in these genes define RCC with different pathological features, gene expression profiles, and outcomes. Negative genetic interactions among cancer genes may indicate that dependencies of cancer gene

action may extend beyond those on tissues. Better understanding of cancer gene dependencies may help to identify weak points in genesis of cancer that can be exploited for anticancer therapeutics.

2.3 Cancer Genome Anatomy Project

CGAP was initiated by the NCI of the USA as an interdisciplinary program to establish the information and technological tools needed for deciphering the molecular anatomy of a cancer cell. One goal of CGAP was to discover new human genes that may be useful in understanding the process of cancer or in monitoring its course. The purpose of CGAP was to develop methods for early detection of cancer, thereby facilitating diagnosis and classification of this disease, assessment of prognosis, and formulation of strategies for cancer prevention. CGAP has been examining the mRNA produced by various types of cancers.

2.4 Human Tumor Gene Index

Human Tumor Gene Index has been established as collaboration between the NCI of the USA, academic institutions, and the industry with the participation of pharmaceutical companies such as GlaxoSmithKline, Merck & Co., Bristol-Myers Squibb, and Genentech. More than 50,000 genes that are active in one or more cancers have been identified. The Tumor Gene Index includes a number of important features such as:

- The index initially focuses on lung, breast, ovarian, prostate, and colon cancer.
- It contains both microdissected and macrodissected tissues.
- Full-length cDNA libraries are constructed from microdissected tissues to provide a template for sequencing projects.
- Gene discovery is linked to pathological analysis of human cancer progression.
- Methods are being developed to assess gene expression from archived tumor tissue.

2.5 Gene Expression Profiling in Cancer

The activity of a gene, so-called gene "expression," means that its DNA is used as a blueprint to produce a specific protein. Only a small number of these genes, about 15,000, are expressed in a typical human cell, but the expressed genes vary from one cell to another. The discovery that eukaryotic genes are not contiguous sequences of DNA but consist of coding sequences (exons) interrupted by

intervening sequences (introns) led to a more complex view of gene expression. The temporal, developmental, typographical, histological, and physiological patterns in which a gene is expressed provide clues to its biological role. Malfunctioning of genes is involved in most diseases including cancer.

Gene expression can be detected by various techniques described in Chap. 6. Most of the cancer research during past few decades has been devoted to the analysis of genes that are expressed differently in tumor cells as compared with their normal counterparts. Intravital microscopy combined with green fluorescent protein (GFP) has provided powerful insight into gene expression in tumors. However, the optical techniques used are plagued by poor axial resolution. Multiphoton laser-scanning microscope can provide high 3D resolution of gene expression and function in deeper regions of tumors. Gene expression studies have shown that many genes that are overexpressed in human cancer cells are specific to a variety of normal tissues, including normal tissues other than those from which the cancer originated. This general property of cancer cells plays a major role in determining the behavior of the cancers, including their metastatic potential.

Functional genomic approaches, such as DNA microarrays and serial analysis of gene expression (SAGE), have enabled researchers to determine the expression level of every gene in a given cell population, which represents that cell population's entire transcriptome. Large-scale gene expression allows simultaneous study of thousands of genes of interest in a specific tissue/tumor of interest, and the ability to identify expression signatures associated with functional phenotypes.

Microarray analysis can be used to search for global signatures of cancer metastases. Certain gene expression profiles correlate with primary versus metastatic cancers, regardless of tumor origin. Some primary tumors already contain a metastatic gene expression signature before they spread to other parts of the body, and this profile can be used to predict patient outcome. Using a cDNA array that contains 10,000–20,000 genes, clinical samples can be analyzed for gene expression differences between benign, local, and metastatic prostatic tissue.

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Chapter 3
Sequencing in Cancer

3.1 Introduction

The genome sequence is an organism's blueprint: the set of instructions dictating its biological traits. The term DNA sequencing refers to methods for determining the exact order of the three billion nucleotide bases—adenine, guanine, cytosine, and thymine—that make up the DNA of the 23 pairs of human chromosomes. In de novo sequencing, short DNA fragments purified from individual bacterial colonies are individually sequenced and assembled electronically into one long, contiguous sequence. This method does not require any preexisting information about the sequence of the DNA. Whole-genome sequencing (WGS) is determination of the primary nucleotide sequence of the entire genome from a single individual. Resequencing using next-generation technologies means determination of variations of DNA sequence in an organism where the nominal sequence is already known. It is often performed using PCR to amplify the region of interest (preexisting DNA sequence is required to design the PCR primers). Resequencing is more relevant for translation into diagnostics and clinical applications.

The advent of large-scale sequencing methods has enabled analyses of the protein-coding parts of cancer genomes to find the mutated genes that cause common human cancers. Unbiased mutation analyses of human cancers have revealed genomic landscapes composed of a few frequently mutated genes alongside a multitude of infrequently mutated genes. These analyses have revealed considerable heterogeneity of mutated genes even among tumors of the same tissue origin (Ali and Sjöblom 2009).

The mutations in individuals' genomes which can lead to cancer are so diverse that many researchers believe that we will have to sequence a patient's tumor DNA before we can effectively treat his disease. Due to growing throughput and shrinking cost, massively parallel sequencing is rapidly becoming an attractive alternative to microarrays for the genome-wide study of gene expression and copy number alterations in primary tumors. The NHGRI-supported, large-scale sequencing centers have begun to tackle the unique challenges associated with using high-throughput

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_3,
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DNA sequencing to characterize tumor genomes. In addition to providing important data on genomic aberrations in select tumor types, this research serves to inform methods and systems development as The Cancer Genome Atlas (TCGA), jointly sponsored by NCI and NHGRI, wraps up its 8-year effort in 2014.

The development of sophisticated genomic analysis sequencing technologies has opened the door to a new era of life-science research, enabling scientists to completely survey entire cancer genomes within individuals. By creating a catalog of both single base changes, or SNPs, and large segments of DNA rearrangements in genomes known as structural variants, researchers hope to one day identify all sources of genetic variation that contribute to cancer. This kind of portrait of the genetic underpinnings of disease will help scientists to lay the groundwork for the molecular events that occur in the generation of individual cancers.

3.2 Sequencing Technologies for Tumor DNA

Collaboration has been established among investigators at the J. Craig Venter Institute and the Johns Hopkins University to assess different technologies for sequencing tumor DNA. This project will analyze the DNA sequence of 37 genes in a collection of 20 glioblastoma tumors. Two sequencing technology platforms will be assessed for their ability to generate useful sequence data from heterogeneous tumor samples: the Sanger sequencing method as implemented on an ABI capillary electrophoresis instrument and the pyrosequencing method as implemented on the sequencing instrument from 454 Life Sciences.

3.2.1 Amplicon Sequencing in Cancer

Human cancer genomes commonly have areas known as amplicons, which are segments of DNA that are repeated many 100s of times and often contain several genes, one of which may cause tumor growth. A publication looked at reports of amplicons in cancer, used a new classification system to rank evidence of their tumor-promoting effects, and identified them as cancer drivers (Santarius et al. 2010). The results identified 77 genes, which are important for the development of cancer, nine of which had not previously been linked to tumor growth: four genes for breast cancer, one for bladder cancer, two for prostate cancer, one for gastric cancer, and one for glioblastoma. Although many cancer genes are easy to detect because they carry clear genetic signatures, amplicons often contain multiple genes, making it difficult to distinguish the driving cancer genes from those that are merely passengers. By linking genome sequencing technologies with the new classification system, the authors hope to be able to continue to identify more genes that contribute to the development of cancer. Most of the genes identified are in biological pathways already known to be involved in cancer development. However, three of the new genes found—YWHAB, YWHAQ,

and CDC6—appear to be in a pathway involved in the initial stages of replication of DNA, a pathway not previously identified in cancer.

Ion AmpliSeq™ Cancer Panel (Ion Torrent), by pairing this 46-gene cancer hotspot panel with the new Ion AmpliSeq Library Kit 2.0, enables detection of rare somatic mutations and enjoys 98 % coverage uniformity and further reductions in strand bias. The Ion AmpliSeq™ Comprehensive Cancer Panel (CCP) reveals tumor mutation profiles and is optimized for use with formalin-fixed paraffin-embedded (FFPE) tissues. This panel enables sensitive, high-coverage detection of rare genetic variants by employing >16,000 primer pairs targeting >400 genes involved in tumor formation. Compared to whole-exome sequencing, Ion AmpliSeq™ CCP requires only 40 ng of input DNA, has a significantly lower price, and provides nearly tenfold better coverage of individual genes, providing better sensitivity and specificity for detecting somatic mutations. CCP delivers exceptional quality, with coverage uniformity and on target bases both greater than 90 %.

3.2.2 Exosome Sequencing

Tumor cells release an abundance of microvesicles containing a selected set of proteins and RNAs. It has been shown that tumor microvesicles (exosomes) also carry DNA, which reflects the genetic status of the tumor, including amplification of the oncogene c-myc (Balaj et al. 2011). Exosome Diagnostics is developing an ultradeep, multiplexed sequencing method using RNA from blood and urine exosome preparations. The method can detect with high-sensitivity rare gene mutations upregulated into exosomes by cancer cells and will form the basis of clinical diagnostic tests for early detection of cancer and as a guide to personalized management of cancer.

3.2.3 Gaining Insights into Mutational Processes

Cancer is driven by mutation. Tobacco smoking is the principal lifestyle exposure that causes cancer, exerting carcinogenicity through >60 chemicals that bind and mutate DNA. A small-cell lung cancer (SCLC) cell line, NCI-H209, was sequenced using massively parallel sequencing technology, to explore the mutational burden associated with tobacco smoking (Pleasance et al. 2010). Multiple mutation signatures that were identified testify to the presence of multiple carcinogens in tobacco smoke and their implication for sequencing. Effects of transcription-coupled repair and a second, more general, expression-linked repair pathway were evident. Identification of a tandem duplication of exons 3–8 of CHD7 in frame, and another two lines carrying PVT1–CHD7 fusion genes, indicates that CHD7 may be recurrently rearranged in this disease. These findings indicate the potential of NGS to provide insights into mutational processes, cellular repair pathways, and gene networks associated with cancer.

3.2.4 Multiplexed Cancer Gene Mutation Analysis

The mass spectrometry-based method known as OncoMap can profile ~400 mutations in 33 oncogenes or tumor suppressor genes. This method has certain limitations, e.g., it can only detect known mutations in a relatively small number of genes and has limited potential for finding small indels and misses chromosomal amplifications and deletions. To overcome these limitations, scientists at the Dana–Farber Cancer Institute (Boston, MA) have developed a massively parallel sequencing strategy for profiling cancer genes from FFPE tumor samples, using it to create barcoded Illumina sequencing libraries. These libraries are then pooled, and Agilent SureSelect capture system is used before sequencing the genes. The scientists tested this approach in ten cancer lines—assessing about 400,000 bases of DNA coding for 138 cancer-related genes that are considered to be clinically actionable. They were able to simultaneously detect SNPs, indels, amplifications, and deletions in the cells. For example, in a breast cancer cell line known as MDA-MB-231, the team detected CDKN1A amplification, along with deletions in JAK2 and CDKN2A—changes that were subsequently validated using microarray analysis. In a pilot study using FPE samples for breast and colon cancer, they identified several mutations that appear to lend themselves to targeted therapeutics including alterations in K-ras, PIK3CA, TSC1, and BRCA1. This method has potential applications for studies of tumor biology as well as for translational analyses.

A multigene NGS test developed and provided by Life Technologies Corporation is in use since 2012 at Cancer Research UK Stratified Medicine laboratories for comparison with existing test methods. Oxford BRC is to measure and report on an expanded panel of 150 genes selected by their investigators and pharmaceutical partners. The genes will include potential new drug targets or biomarkers for efficacy of existing drugs or those in development. A new clinical trial model could emerge in which multigene testing aims to make trial enrolment less of a trial-and-error process. With availability of standardized genomic testing, one diagnostic test will enable a physician to recommend several trials that may be suitable for a patient. At the same time, pharmaceutical companies should be able to start their studies more quickly and will be able to review the genetic profile of patient cohorts to better understand the genetic factors associated with favorable or unfavorable drug response. This will facilitate the development of personalized cancer therapies.

3.2.5 NGS-Based Molecular Profiling of Cancer in FFPE Specimens

Foundation Medicine Inc. has developed NGS-based molecular profiling of cancer in FFPE specimens with an aim to bring comprehensive cancer genome analysis to routine care of cancer patients. A study was conducted on colorectal cancer, NSCLC, and melanoma specimens by performing paired-end sequencing on the Illumina

HiSeq™ 2000 platform for 2,574 exons representing 176 genes captured using Agilent SureSelect™ baits. Comprehensive variant profiles were generated for each sample, including base substitutions, insertions/deletions, copy number alterations, and targeted rearrangements. Results showed that the test identified a total of 214 driver mutations (including multiple mutations in well-known cancer genes such as TP53, STK11, APC, CDH1, ATM, GNAS, SMAD4, PIK3CA, KIT, MDM2, and CDKN2A), of which only 37 (17.3 %) could have been detected by conventional hotspot analyses. Results of the study were 100 % concordant with conventional, single gene analysis previously reported by commercial reference laboratories for BRAF, K-ras, and EGFR. More than 50 % of specimens studied had mutations which may be useful for decision-making in management of cancer patients.

3.2.6 Paired-End Sequencing

Characterization of breakpoints in disease-associated balanced chromosome rearrangements (DBCRs), which disrupt or inactivate specific genes, has facilitated the molecular elucidation of a wide variety of genetic disorders and cancer. However, conventional methods for mapping chromosome breakpoints, such as fluorescent in situ hybridization (FISH) with dye-labeled bacterial artificial chromosome (BAC) clones, are laborious and time-consuming and usually do not provide sufficient resolution for definite identification of the disrupted gene. The efficiency of breakpoint mapping has improved considerably by combination of DNA array hybridization with chromosome sorting. However, this can only be applied when the physical properties of the derivative chromosomes allow them to be flow sorted. For characterizing the breakpoints in all types of balanced chromosome rearrangements more efficiently and more accurately, massively parallel sequencing has been performed using Illumina 1G analyzer and SOLiD systems to generate short sequencing reads from both ends of DNA fragments. This method was applied to four different DBCRs, including two reciprocal translocations and two inversions (Chen et al. 2010). By identifying read pairs spanning the breakpoints, the authors were able to map the breakpoints to a region of a few hundred base pairs that could be confirmed by subsequent PCR amplification and Sanger sequencing of the junction fragments. These results show the feasibility of paired-end sequencing of systematic breakpoint mapping and gene finding in patients with disease-associated chromosome rearrangements.

Paired-end sequencing is emerging as a key technique for assessing genome rearrangements and structural variation on a genome-wide scale. This technique is particularly useful for detecting copy-neutral rearrangements, such as inversions and translocations, which are common in cancer and can produce novel fusion genes. This approach will be useful in calibrating future cancer sequencing efforts, particularly large-scale studies of many cancer genomes that are enabled by next-generation sequencing technologies (Bashir et al. 2008).

Recurrent gene fusions, typically associated with hematological malignancies and rare bone and soft tissue tumors, have recently been described in common solid

tumors. An integrative analysis of high-throughput long- and short-read transcriptome sequencing of cancer cells has been used to "re-discover" the BCR–ABL1 gene fusion in a chronic myelogenous leukemia cell line and the TMPRSS2–ERG gene fusion in prostate cancer cell line and tissues (Maher et al. 2009). High-throughput sequencing is opening up an important class of cancer-related mutations for comprehensive characterization.

Genome-wide paired-end massively parallel DNA sequencing has been used to rapidly map translocation breakpoints (Slade et al. 2010). This method was applied for fine mapping of a de novo t(5;6)(q21;q21) translocation in a child with bilateral, young-onset Wilms tumor. This involved genome-wide paired-end sequencing of approximately six million randomly generated ~3 kb fragments from constitutional DNA containing the translocation and led to identification of six fragments in which one end mapped to chromosome 5 and the other to chromosome 6. PCR assays were designed that amplified across the rearrangement junction to characterize the breakpoints at sequence level resolution. The 6q21 breakpoint transects and truncates HACE1, an E3 ubiquitin-protein ligase that has been implicated as a somatically inactivated target in Wilms tumorigenesis. These data indicate that constitutional disruption of HACE1 likely predisposes to Wilms tumor. However, HACE1 mutations are rare and therefore can only make a small contribution to Wilms tumor incidence. Nevertheless, this study demonstrates the usefulness of genome-wide paired-end sequencing in the delineation of apparently balanced chromosomal translocations, for which it is likely to become the method of choice.

Paired-end sequencing strategy has been used to identify somatic rearrangements in breast cancer genomes (Stephens et al. 2009). There are more rearrangements in some breast cancers than previously appreciated. This study has demonstrated the ability of next-generation sequencing technologies to detect chromosomal rearrangements at a level that previous sequencing technologies and cytogenetics could not do. The study indicates that whole-genome short-read sequencing technologies are becoming increasingly important for identifying cancer-causing genes that could eventually lead to better diagnosis and treatment.

3.2.7 RNA-Seq to Study Cancer Transcriptome

RNA-Seq uses NGS to study the transcriptome—the set of all RNA molecules—at a nucleotide level. It evaluates cDNA, which is synthesized from an mRNA template. RNA-Seq, a massively parallel method, improves on microarray technology because it sequences transcriptomes, which are easier to sequence than genomes due to the smaller size of the section of DNA that code for protein. RNA-Seq offers the ability to detect somatic mutations and accurately measure allele-specific expression. To investigate these advantages, a novel strand-specific RNA-Seq was applied to tumors and matched normal tissue from patients with oral squamous cell carcinomas (Tuch et al. 2010). In order to better understand the genomic determinants of the gene expression changes observed, the tumor and normal genomes of one of these

patients were also sequenced. By comparing the transcriptomic perturbations observed in one patient to his underlying normal and tumor genomes, the allelic imbalance in the tumor has been associated with copy number mutations, which are strongly associated with changes in transcript abundance. These results support a model in which allele-specific deletions and duplications drive allele-specific changes in gene expression in the developing tumor that could reveal important therapeutic targets. The information provided by studies like this will help to clarify the molecular basis for cancer development and thus provide valuable insight into developing therapeutic strategies for treating cancer.

3.2.8 Sequencing Cancer Cell Lines

U87MG, a commonly studied glioblastoma cell line, has been decoded as a model of broad cancer genome sequencing (Clark et al. 2010). The authors generated greater than 30× genomic sequence coverage using a novel 50-base mate-paired strategy with a 1.4 kb mean insert library. All data were aligned using a custom designed tool called BFAST, allowing optimal color space read alignment and accurate identification of DNA variants. The aligned sequence reads and mate-pair information identified 35 interchromosomal translocation events, 1,315 structural variations, 191,743 small insertions and deletions (indels), and 2,384,470 single-nucleotide variations (SNVs). Among these observations, the known homozygous mutation in PTEN was robustly identified, and genes involved in cell adhesion were overrepresented in the mutated gene list. Data were compared to 219,187 heterozygous SNPs assayed by Illumina 1M Duo genotyping array to assess accuracy: 93.83 % of all SNPs were reliably detected at filtering thresholds that yield greater than 99.99 % sequence accuracy. Protein-coding sequences were disrupted predominantly in this cancer cell line due to small indels, large deletions, and translocations. In total, 512 genes were homozygously mutated, including 154 by SNVs, 178 by small indels, 145 by large microdeletions, and 35 by interchromosomal translocations to reveal a highly mutated cell line genome. Of the small homozygously mutated variants, 8 SNVs and 99 indels were novel events not present in dbSNP. These data demonstrate that routine generation of broad cancer genome sequence is possible outside of genome centers. The sequence analysis of U87MG provides an unparalleled level of mutational resolution compared to any cell line to date.

3.2.9 Sequencing for Studying Chromothripsis in Cancer

Although somatic mutations driving cancer usually accumulate gradually over time, they could also arise in a single catastrophic event of chromosome shattering termed chromothripsis whereby tens to hundreds of genomic rearrangements occur in a single cellular crisis (Stephens et al. 2011). This phenomenon was demonstrated by

using high-throughput sequencing and microarrays. The authors documented chromothripsis in a number of human cancers and showed that several oncogenic lesions can emerge from one genomic crisis. Results from analyses of hundreds of cancer cell lines indicate that approximately 2–3 % of all cancers and about a quarter of bone cancers involve chromothripsis. While some cells with catastrophic chromosomal damage undergo apoptosis and die, others can stitch together chromosomal fragments and then go on not only to survive but also to gain a selective advantage that ultimately leads to cancer. This is consistent with the apparent rapidity with which cancer appears in some cases.

The discovery stems from the authors' efforts to characterize rearrangements in chronic lymphocytic leukemia (CLL). Using the Illumina Genome Analyzer II, the researchers did massively parallel, paired-end sequencing to look for chromosomal rearrangements in ten CLL samples collected from as many different individuals. One of these individuals carried not only a smattering of focal point changes and rearrangements on chromosomes 1, 12, 13, and 15 but also a cluster of 42 rearrangements concentrated on one arm of chromosome 4, a pattern that was strikingly different from the genome-wide changes previously reported for breast, lung, and pancreatic cancers. Given the patterns detected, the team argues that it is extremely unlikely that these genetic changes accumulated in the cells over time. Instead, the nature and localization of these rearrangements point to rapid rearrangements in the genome. Although much of the genome is quiet in cells that have undergone chromothripsis, these cells typically have one chromosome, chromosomal arm, or region of a chromosome that is radically rearranged. Rearrangements involving one or a few chromosomes crisscross back and forth across involved regions. More research is needed to determine the cause of chromothripsis. It might be a consequence of exposure to ionizing radiation and/or other environmental exposures. Faced with hundreds of DNA breaks, the cell's DNA repair machinery attempts to rescue the genome. The resultant hodgepodge bears little resemblance to its original structure, and the genomic disruption has wholesale and potentially oncogenic effects.

Chromothripsis has been associated with aggressive cancer, but it also has a role in the genesis and progression of benign tumors. WGS and gene expression profiling of uterine leiomyomas have shown that chromosome shattering and reassembly resembling chromothripsis is a major cause of chromosomal abnormalities in uterine leiomyomas (Mehine et al. 2013). The authors proposed that tumorigenesis occurs when tissue-specific tumor-promoting changes are formed through these events.

3.2.10 Sequencing of Complex Human Cancer Genomes

Human cancers often carry many somatically acquired genomic rearrangements, but conventional strategies for characterizing these are laborious and low throughput and have low sensitivity or poor resolution. Massively parallel sequencing has been used to generate sequence reads from both ends of short DNA fragments derived from the genomes of two individuals with lung cancer (Campbell et al.

2008). By investigating read pairs that did not align correctly with respect to each other on the reference human genome, germline structural variants and somatic rearrangements were characterized to the base-pair level of resolution. The patterns of germline and somatic rearrangement were markedly different. The results demonstrate the feasibility of systematic, genome-wide characterization of rearrangements in complex human cancer genomes, raising the prospect of a new harvest of genes associated with cancer using this strategy.

3.2.11 Sequencing Single Cells to Study Evolution of Cancer

In cancers, where genetic heterogeneity is common, CNVs cannot resolve mixed populations of cells, and important information that would be useful for reconstructing evolutionary history may be lost. Accurate quantification of genomic copy number within an individual nucleus is feasible with flow-sorted nuclei, WGA, and NGS, and it is possible to make inferences about the evolution and spread of cancer by examining multiple cells from the same cancer (Navin et al. 2011). The authors of this study applied single-nucleus sequencing to investigate tumor population structure and evolution in two human breast cancer cases. Analysis of 100 single cells from a polygenomic tumor revealed three distinct clonal subpopulations that probably represent sequential clonal expansions. Additional analysis of 100 single cells from a monogenomic primary tumor and its liver metastasis indicated that a single clonal expansion formed the primary tumor and seeded the metastasis. Patterns in the metastatic sample indicate that cancers cropping up at secondary sites in the body carry many of the same copy number patterns of the original tumor. In contrast to gradual models of tumor progression, these data indicate that tumors grow by mutational spurts rather than a gradual snowballing of mutations. In addition, both primary tumors tested contained “pseudodiploid cells” harboring a host of genetically diverse rearrangements. These rearrangements did not seem to overlap from one pseudodiploid cell to the next and did not show up in aneuploid cell populations in the tumor samples. These pseudodiploid cells might represent a population of cancer precursor cells that spur some of the genomic diversity, which eventually leads to cancer development.

3.2.12 Sequencing for Assessing Resistance to Anticancer Therapy

Resistance to anticancer therapies is an important cause of treatment failure. RAF inhibition in BRAF-mutant melanoma is an example of the promise and challenge of many targeted anticancer drugs; although response rates are high, resistance invariably develops. Tumor mutation profiling has been used to characterize resistance in the clinical setting. As a proof of principle, massively parallel sequencing

of cancer genes was performed in a tumor obtained from a patient with melanoma who developed resistance to PLX4032 after an initial dramatic response (Wagle et al. 2011). The resulting profile identified an activating mutation at codon 121 in the downstream kinase MEK1 that was absent in the corresponding pretreatment tumor. The MEK1C121S mutation was shown to increase kinase activity and confer robust resistance to both RAF and MEK inhibition in vitro. Thus, MEK1C121S or functionally similar mutations are predicted to confer resistance to combined MEK/RAF inhibition. These results provide an insight into mechanisms of acquired resistance to kinase inhibition.

3.3 Cancer Genome Atlas

TCGA is a coordinated effort to accelerate our understanding of the molecular basis of cancer through the application of genome analysis technologies, including large-scale genome sequencing (http://cancergenome.nih.gov/). A component of TCGA Pilot Project will be high-throughput genomic sequencing. This activity will be conducted by the following Genome Sequencing Centers that have extensive experience in large-scale genomic DNA sequencing:

- Broad Institute Sequencing Platform, Broad Institute of MIT and Harvard, Cambridge, MA
- Washington University Genome Sequencing Center, Washington University School of Medicine, St. Louis, MO
- Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX

The Tumor Sequencing Project (TSP) Consortium is a collaboration among participants at the Baylor College of Medicine Human Genome Sequencing Center, the Broad Institute Genome Sequencing Platform, the Dana–Farber Cancer Institute, the Memorial Sloan–Kettering Cancer Center, the Genome Sequencing Center and Siteman Cancer Center at Washington University, the MD Anderson Cancer Center, and the University of Michigan Medical Center. The TSP will pilot approaches to large-scale identification of genomic changes in tumors and aims to sequence the exonic regions of 1,000 genes in almost 200 specimens of adenocarcinoma of the lung, as well as use high-density SNP genotyping arrays for high-resolution identification of changes in chromosomal copy number.

International Cancer Genome Consortium (ICGC) (http://www.icgc.org/) is planning to sequence genomes of 20,000 cancer patients spread across 50 tumor types and subtypes so that any single tumor type will be sequenced with sufficient statistical power to identify all significant underlying variants. This will provide the investigators with a new approach to explore the genetic basis of the particular cancers they are studying. They will be able to investigate associations between the genetic changes observed in cancer genomes and compare them with normal genomes. This completeness of data, i.e., developing a comprehensive list of changes in thousands of genomes, will enable the molecular definition of cancer

subtypes according to the type of change and affected pathways. This would lead to the discovery of hundreds of highly informative diagnostic and prognostic biomarkers, with applications in predictive personalized disease prevention, earlier diagnosis, and improved treatments, while minimizing drug resistance and harmful side effects. Indications for use of some existing anticancer drugs can be redefined. With evolving cost-effective sequencing technologies, the cost of such studies, currently estimated to be hundreds of millions of dollars, would drop considerably.

3.4 Sequencing of Tumors of Various Organs/Systems

3.4.1 Sequencing of Brain Tumors

There is significant interest in new sequencing-based technologies that map genetic and epigenetic alterations in brain tumors comprehensively and at high resolution. Members of the PedBrain Tumor Consortium plan to sequence DNA from matched tumor–normal samples to about 30 times coverage with the aim of uncovering the genetic underpinnings of pediatric brain cancer. In the process, they hope to find clues for improving pediatric cancer treatments and diagnostics. Several sequencing projects are underway with this aim. Washington University (St. Louis, MO) and St. Jude Children's Research Hospital have their own pediatric cancer genome sequencing projects, aimed at sequencing more than 600 pediatric cancer genomes over 3 years to find genetic and epigenetic patterns in these cancers. The German Cancer Research Center and GATC Biotech are collaborating to sequence children's brain tumors for a project being done within the ICGC.

3.4.1.1 Sequencing for Genetic Alterations in Gliomas

Variants at 8q24.21 have been shown to be associated with glioma development. By means of tag SNP genotyping and imputation, pooled NGS using long-range PCR, and subsequent validation of SNP genotyping, the authors of a publication identified seven low-frequency SNPs at 8q24.21 that were strongly associated with glioma risk (Jenkins et al. 2012). The most strongly associated SNP, rs55705857, remained highly significant after individual adjustment for the other top six SNPs and two previously published SNPs. After stratifying by histological and tumor genetic subtype, the most significant associations of rs55705857 were with oligodendroglial tumors and gliomas with mutant IDH1 or IDH2. Strong associations were observed for astrocytomas with mutated IDH1 or IDH2 (grades 2–4) but not for astrocytomas with wild-type IDH1 and IDH2. The conserved sequence block that includes rs55705857 is consistently modeled as a miRNA.

Pediatric low-grade gliomas (PLGGs) are among the most common solid tumors in children but, apart from BRAF kinase mutations or duplications in specific subclasses, few genetic driver events are known. Diffuse PLGGs comprise a set of

uncommon subtypes that exhibit invasive growth and are therefore especially challenging clinically. High-resolution CNV analysis performed on formalin-fixed, paraffin-embedded diffuse PLGGs has been done to identify recurrent alterations (Ramkissoon et al. 2013). Diffuse PLGGs exhibited fewer such alterations than adult low-grade gliomas, but several significantly recurrent events were identified. The most significant event, 8q13.1 gain, was observed in 28 % of diffuse astrocytoma grade IIs and resulted in partial duplication of the transcription factor MYBL1 with truncation of its C-terminal negative-regulatory domain. A similar recurrent deletion–truncation breakpoint was identified in two angiocentric gliomas in the related gene v-myb avian myeloblastosis viral oncogene homolog (MYB) on 6q23.3. WGS of a MYBL1-rearranged diffuse astrocytoma grade II demonstrated MYBL1 tandem duplication and few other events. Truncated MYBL1 transcripts identified in this tumor induced anchorage-independent growth in 3T3 cells and tumor formation in nude mice. Truncated transcripts were also expressed in two additional tumors with MYBL1 partial duplication. These results define clinically relevant molecular subclasses of diffuse PLGGs and highlight a potential role for the MYB family in the biology of low-grade gliomas.

WGS to identify multiple new genetic alterations involving BRAF, RAF1, FGFR1, MYB, MYBL1, and genes with histone-related functions, including H3F3A and ATRX, in PLGGs revealed only a single non-silent somatic alteration in 62 % of tumors (Zhang et al. 2013). Intragenic duplications of the portion of FGFR1 encoding the tyrosine kinase domain (TKD) and rearrangements of MYB were recurrent and mutually exclusive in 53 % of grade II diffuse PLGGs. Transplantation of Trp53-null neonatal astrocytes expressing FGFR1 with the duplication involving the TKD into the brains of nude mice generated high-grade astrocytomas with short latency and 100 % penetrance. FGFR1 with the duplication induced FGFR1 autophosphorylation and upregulation of the MAPK/ERK and PI3K pathways, which could be blocked by specific inhibitors. Focusing on the therapeutically challenging diffuse PLGGs, this study has discovered genetic alterations and potential therapeutic targets across the entire range of PLGGs.

3.4.1.2 Sequencing for Genetic Alterations in Medulloblastoma

Medulloblastoma is an aggressively growing tumor, arising in the cerebellum or medulla/brain stem. It is the most common malignant brain tumor in children and shows tremendous biological and clinical heterogeneity. Despite recent treatment advances, approximately 40 % of children experience tumor recurrence, and 30 % will die from their disease. To identify the genetic alterations in medulloblastoma, a search was made for CNVs using high-density microarrays and sequencing of all known protein-coding genes and miRNA genes using Sanger sequencing in a set of 22 tumors (Parsons et al. 2011). On average, each tumor was found to have 11 gene alterations, fewer by a factor of 5–10 than in the adult solid tumors that have been sequenced to date. In addition to alterations in the Hedgehog and Wnt pathways, this study led to the discovery of genes not previously known to be altered in

medulloblastomas; most notably, inactivating mutations of the histone-lysine *N*-methyltransferase genes MLL2 or MLL3 were identified in 16 % of patients. These results demonstrate key differences between the genetic landscapes of adult and childhood cancers, highlight dysregulation of developmental pathways as an important mechanism underlying medulloblastomas, and identify a role for a specific type of histone methylation in human tumorigenesis.

An integrative deep sequencing analysis of 125 tumor–normal pairs was conducted as part of the ICGC PedBrain Tumor Project (Jones et al. 2012). Tetraploidy was identified as a frequent early event in Group 3 and 4 tumors, and a positive correlation between patient age and mutation rate was observed. Several recurrent mutations were identified, both in known medulloblastoma-related genes (CTNNB1, PTCH1, MLL2, SMARCA4) and in genes not previously linked to this tumor (DDX3X, CTDNEP1, KDM6A, TBR1), often in subgroup-specific patterns. RNA sequencing confirmed these alterations and revealed the expression of the first medulloblastoma fusion genes identified. Chromatin modifiers were frequently altered across all subgroups. These findings enhance our understanding of the genomic complexity and heterogeneity underlying medulloblastoma and provide several potential targets for new therapeutics, especially for Group 3 and 4 patients.

3.4.2 Sequencing of Breast Cancer

Current approaches typically rely on PCR amplification followed by Sanger sequencing of individual exons for finding point mutations and indels, followed by another test to detect larger exonic deletions and duplications. NGS of breast cancer genomes reveals insight into tumor heterogeneity and how it can contribute to future breast cancer classification and management.

3.4.2.1 BRCA Mutations

Inherited loss-of-function mutations in the tumor suppressor genes BRCA1, BRCA2, and multiple other genes predispose to high risks of breast and/or ovarian cancer. Cancer-associated inherited mutations in these genes are collectively quite common, but individually rare. Genetic testing for BRCA1 and BRCA2 mutations (Myriad Genetics) has become an integral part of clinical practice, but testing is generally limited to these two genes and to women with severe family histories of breast or ovarian cancer. It may miss some women with paternally inherited BRCA1/BRCA2 mutations or mutations in other cancer-related genes. NewGene Inc.'s breast cancer genetic test, unlike Myriad's BRACAnalysis, which is PCR based, uses next-generation sequencing technology that results in faster turnaround times and lower costs compared to other technologies. This will lead to improved access to a breast cancer genetic test with clinical use for patients. The test is based on full gene sequencing of the BRCA1 and BRCA2 genes, so it is not targeting specific

mutations. NewGene uses the Roche 454 GS-FLX platform for pyrosequencing. Unlike traditional Sanger sequencing, which involves looking at individual segments of a gene one segment at a time and one patient at a time, pyrosequencing enables the investigation of genes of interest in multiple patients in the same run and with multiple gene fragments in the same run. Thus, NewGene can look at 20,000 fragments in one run in contrast to one fragment per run allowed by Sanger sequencing-based methods. Because each patient requires about 100 fragments to be sequenced, the increase in the number of patients that can be investigated in a single run and the improvement in throughput achieved by this technology are significant. Test results using this technology can be achieved in as little as 4 weeks.

A genomic assay has been developed to capture, sequence, and detect all mutations in 21 genes, including BRCA1 and BRCA2, with inherited mutations that predispose to breast or ovarian cancer (Walsh et al. 2010). Constitutional genomic DNA from subjects with known inherited mutations, ranging in size from 1 to >100,000 bp, was hybridized to custom oligonucleotides and then sequenced using a genome analyzer. Analysis was carried out blind to the mutation in each sample. Together, these sequences account for roughly a million bases of DNA per individual. Average coverage was >1,200 reads per base pair. After filtering sequences for quality and number of reads, all single-nucleotide substitutions, small insertion and deletion mutations, and large genomic duplications and deletions were detected. There were zero false-positive calls of nonsense mutations, frameshift mutations, or genomic rearrangements for any gene in any of the test samples. This approach enables widespread genetic testing and personalized risk assessment for breast and ovarian cancer. By allowing comprehensive parallel testing of multiple cancer susceptibility genes, it will be possible to confidently identify the fraction of women with breast or ovarian cancer who carry a germline alteration in a cancer susceptibility allele and the characteristics of the tumors of patients' inherited mutations in various genes. The whole procedure can be done for less than $1,500 per sample. New moderate- to high-risk breast and/or ovarian cancer-associated genes will likely be incorporated into the method as they are discovered.

3.4.2.2 Circulating Nucleic Acids as Biomarkers of Cancer

Circulating nucleic acids (CNA), isolated from serum or plasma, are increasingly recognized as biomarkers of cancer. Recently developed NGS provides high numbers of DNA sequences to detect the trace amounts of unique serum biomarkers associated with breast carcinoma. Serum CNA of women with ductal carcinoma was extracted and sequenced on a 454/Roche high-throughput GS-FLX platform and compared with healthy controls and patients with other medical conditions (Beck et al. 2010). Breast cancer was accurately detected at a diagnostic specificity level of 95 % with a calculated sensitivity of 90 %. Identification of specific breast cancer-related CNA sequences provides the basis for the development of a serum-based routine laboratory test for breast cancer screening and monitoring. This is in development by Chronix Biomedical.

3.4.2.3 Deep Sequencing of miRNA for Signatures of Invasiveness

The transition from ductal carcinoma in situ to invasive ductal carcinoma is a key event in breast cancer progression that is still not well understood. To discover the miRNAs regulating this critical transition, biopsies were used from invasive ductal carcinoma, from ductal carcinoma in situ, and from normal breast (Volinia et al. 2012). The miRNA signatures were selected from a recently published deep sequencing dataset (Farazi et al. 2011). The miRNA profile established for the normal breast to ductal carcinoma in situ transition was largely maintained in the in situ to invasive ductal carcinoma transition. Nevertheless, a 9-miRNA signature was identified that differentiated invasive from in situ carcinoma. Specifically, let-7d, miR-210, and miR-221 were downregulated in the in situ and upregulated in the invasive transition, thus featuring an expression reversal along the cancer progression path. Additionally, microRNAs were identified for overall survival and time to metastasis. Five noncoding genes were associated with both prognostic signatures—miR-210, miR-21, miR-106b*, miR-197, and let-7i, with miR-210 as the only one also involved in the invasive transition. To pinpoint critical cellular functions affected in the invasive transition, we identified the protein-coding genes with inversely related profiles to miR-210: BRCA1, FANCD, FANCF, PARP1, E-cadherin, and Rb1 were all activated in the in situ and downregulated in the invasive carcinoma. Additionally, we detected differential splicing isoforms with special features, including a truncated EGFR lacking the kinase domain and overexpressed only in ductal carcinoma in situ.

3.4.2.4 Sequencing of Breast Cancer Metastases

Massively parallel DNA sequencing technologies provide an unprecedented ability to screen entire genomes for genetic changes associated with tumor progression. Genomic analyses of four DNA samples from an African-American patient with basal-like breast cancer—peripheral blood, the primary tumor, a brain metastasis, and a xenograft derived from the primary tumor—have been reported (Ding et al. 2010). The metastasis contained two de novo mutations and a large deletion not present in the primary tumor and was significantly enriched for 20 shared mutations. The xenograft retained all primary tumor mutations and displayed a mutation enrichment pattern that resembled the metastasis. Two overlapping large deletions, encompassing CTNNA1, were present in all three tumor samples. The differential mutation frequencies and structural variation patterns in metastasis and xenograft compared with the primary tumor indicate that secondary tumors may arise from a minority of cells within the primary tumor.

Basal-like breast cancer was selected for the study because it is aggressive and tends to affect younger African-American women. Moreover, it is estrogen receptor (ER) negative and responds poorly to chemotherapy. During their analyses of these genomes, the team identified 50 point mutations and small insertions or deletions that were present in one of the tumor genomes but not matched normal tissue, including 48 mutations found in all three tumors. Of the 48 point mutations and

small indels present in all three tumors, 20 were significantly enriched in the metastatic tumor, reflecting the fact that the metastatic tumor is made up of a subset of cells that have survived treatment. The affected genes included CSMD1, a gene shown to be mutated in some colorectal, head, and neck cancers that has also been linked to survival in invasive ductal breast cancer, JAK2 and NRK, genes mutated in some other breast cancers, and TP53, another known cancer-related gene. These results suggest the same genes are often mutated in cancers turning up at various sites in the body. Additional cancer genome analyses may eventually yield clues about developing site-agnostic treatments. The similarity between the mouse and human tumors suggests that mouse models can be valid preclinical surrogates of metastatic disease to evaluate new cancer drugs.

3.4.2.5 Triple-Negative Breast Cancer

Subtypes of breast cancer are generally diagnosed based upon the presence or lack of three receptors that are known to fuel most breast cancers: progesterone receptors (PR), ER, and HER2. Unfortunately, none of these receptors are found in women with triple-negative breast cancer (TNBC), i.e., the offending tumor is ER-negative, PR-negative, and HER2-negative and does not respond to receptor targeted treatments.

WGS has revealed previously unreported mutations in metastatic TNBC. Somatic genomic alterations in these advanced tumors, particularly those that might guide targeted therapies, have been cataloged following initial analyses of WGS and transcriptome sequencing data from prospective metastatic mTNBC (Craig et al. 2013). In a sample of 14 tumors from ethnically diverse metastatic TNBC patients, the researchers found significant mutations and other changes in more than a dozen genes through WGS performed on Life Technologies' SOLiD™ 4.0. The most frequently mutated gene among the tumors (7 of 14) was the TP53 tumor suppressor, and aberrations were observed in additional tumor suppressor genes including CTNNA1, which was detected in 2 of 6 African-American patients (who typically have more aggressive and treatment-resistant disease). Alterations were also seen in the ERBB4 gene, known to be involved in mammary-gland maturation during pregnancy and lactation, but not previously linked to mTNBC. RNA sequencing revealed consistent overexpression of the FOXM1 gene, when tumor gene expression was compared to nonmalignant breast samples. Using an outlier analysis of gene expression comparing one cancer to all the others, the authors detected expression patterns unique to each patient's tumor. Integrative DNA/RNA analysis provided evidence for deregulation of mutated genes. Finally, molecular alterations in several cancers supported targeted therapeutic intervention on clinical trials with known inhibitors, particularly for alterations in the RAS/RAF/MEK/ERK and PI3K/AKT/MTOR pathways. In conclusion, whole-genome and transcriptome profiling of mTNBC have provided insights into somatic events occurring in this difficult-to-treat cancer. These genomic data have guided patients to investigational treatment trials and provide hypotheses for future trials in this irremediable cancer. Genome sequencing

will eventually become a standard tool for oncologists, enabling them to tailor therapies to the unique genetic profiles of each of their patients.

3.4.2.6 Whole-Genome Sequencing in Breast Cancer

Genomic Health, using Illumina's NGS technology, has completed sequencing of the whole human transcriptome in FFPE tumor and normal breast tissue samples and found hundreds of differences in both coding and noncoding transcripts between the two sample populations. Analysis showed that prognostic significance of these differences was modest. Further evaluation of these transcripts by gene-set analysis produced a group that was rich in prognostic genes. RT-PCR assays were designed for a number of noncoding transcripts and were used to screen breast cancer specimens revealing an association between specific genes and some noncoding RNAs with risk recurrence of breast cancer.

A genome-wide functional profiling approach was used to identify multiple genes that confer resistance or sensitivity to tamoxifen. Combining whole-genome shRNA screening with massively parallel sequencing, the impact of more than 56,670 RNA interference reagents targeting 16,487 genes on the cellular response to tamoxifen was profiled (Mendes-Pereira et al. 2012). This screen, along with subsequent validation experiments, identified a compendium of genes whose silencing causes tamoxifen resistance (including BAP1, CLPP, GPRC5D, NAE1, NF1, NIPBL, NSD1, RAD21, RARG, SMC3, and UBA3) and also a set of genes whose silencing causes sensitivity to this endocrine agent (C10orf72, C15orf55/NUT, EDF1, ING5, K-ras, NOC3L, PPP1R15B, RRAS2, TMPRSS2, and TPM4). Multiple individual genes, including NF1, a regulator of RAS signaling, also correlate with clinical outcome after tamoxifen treatment.

3.4.3 Sequencing of Colorectal Cancer

TCGA project plans to profile genomic changes in 20 different cancer types and has now presented results from multidimensional analyses of human colorectal carcinoma (CRC). CRC is an important contributor to cancer mortality and morbidity. The distinction between the colon and the rectum is largely anatomical, but it has both surgical and radiotherapeutic management implications and it may have an impact on prognosis. Most investigators divide CRC biologically into those with microsatellite instability (MSI), which are located primarily in the right colon and are frequently associated with the CpG island methylator phenotype as well as hypermutation, and those that are microsatellite stable but chromosomally unstable.

Previous investigations have uncovered several critical genes and pathways that are important in the initiation and progression of CRC. These include the WNT, RAS–MAPK, PI3K, TGF-β, P53, and DNA mismatch repair pathways. Large-scale sequencing analyses have identified numerous recurrently mutated genes and a

recurrent chromosomal translocation, but a fully integrated view of the genetic and genomic changes and their significance for CRC tumorigenesis was lacking. Genomic patterns that have now been uncovered in CRC, including samples originating at sites in either the colon or the rectum, reveal genomic profiles that are similar to those present in tumors at each site (The Cancer Genome Atlas Network 2012). By doing sequencing, CNV analyses, and/or methylation profiling on almost 300 CRC samples, the team narrowed in on key genes and pathways that tend to be altered in CRC. For the new analysis, researchers used SOLiD or Illumina sequencing platforms to sequence the exomes of 224 tumor–normal pairs to an average depth of >20× over 80 % or more of the coding sequences targeted. With the Illumina HiSeq 2000, they also did low-coverage WGS on 97 of the tumors and matched normal samples. The transcriptional analysis was expanded further through RNA sequencing and miRNA sequencing experiments. The data presented provide a useful resource for understanding CRC and identifying possibilities for treating it in a targeted way. Although it may take years to translate this foundational genetic data on CRC into new therapeutic strategies and surveillance methods, this genetic information will be the springboard for determining what will be clinically effective against CRC. A subset of the CRC, most often tumors showing up in the right or ascending colon, had unusually high mutation levels. Approximately 16 % of the tumors could be classified as hypermutated, containing a median of 728 predicted somatic mutations apiece.

More than 75 % of these hypermutated samples showed enhanced methylation levels and MSI. As the survival rate of patients with high MSI-related cancers is better and these cancers are hypermutated, mutation rate may be a better prognostic indicator.

From their genome sequence data, researchers tracked down several suspected translocation events involving bits of sequence from different chromosomes. For example, 3 of the 97 tumors assessed by low-coverage genome sequencing contained a fusion linking the first exons of the chromosome 11 gene NAV2 to part of the chromosome 2 gene TCF7L1, which codes for a component in the WNT pathway, a known contributor to CRC. Almost all of the tumors from both the hypermutated and the non-hypermutated groups included mutations expected to boost the activity of the WNT signaling pathway and to curb signaling via the TGF-β pathway, changes that are expected to produce an increase in the activity of the myc proto-oncogene. These findings fit with early reports suggesting that compounds targeting that pathway may be effective against some CRCs. Possible therapeutic approaches to CRC included WNT signaling inhibitors and small-molecule β-catenin inhibitors, which are showing initial promise. Other commonly affected pathways included the RTK–RAS, MAP kinase, TP53, and PI3 kinase pathways, which point to potential targets for new CRC treatments. Approximately 5 % of the CRC tumors studied had extra copies of a gene, ERBB2, as do many breast cancer tumors. The drug Herceptin, which is effective for breast cancer patients with too many ERBB2 genes, might also help CRC patients with the same aberration. Clinical trials have been proposed to test the effects of Herceptin in these CRC patients. Approximately 15 % of CRCs had a mutation in a gene BRAF that is also

often mutated in melanoma. A drug approved for melanoma blocks the function of BRAF gene product, but it has not worked in CRC patients.

3.4.4 Sequencing of Head and Neck Cancer

3.4.4.1 NGS for Detection of HPV Sequences in Carcinoma of Oropharynx

Human papillomavirus (HPV) infection in cases of squamous cell carcinoma of the oropharynx is a strong predictive and prognostic biomarker. Use of NGS can provide a novel method for the detection of HPV in DNA isolated from FFPE tissues from head and neck cancer patients, the viral subtype involved, and a direct readout of viral load (Conway et al. 2012). A lower level of chromosome instability was detected in HPV-positive tumors compared to HPV-negative tumors, as observed in previous studies. Specificity of HPV detection by sequencing compared to traditional detection methods using either PCR or p16 immunohistochemistry was 100 %. Sensitivity was 50 % when either compared to PCR or 75 % when compared to p16. In addition, the ability of NGS to detect other HPV subtypes that would not have been detected by traditional methods was demonstrated. This method can be applied to any tumor and any virus. It also provides a tumor genomic copy number karyogram. Thus, the use of NGS for the detection of HPV in cancer provides a multiplicity of data with clinical significance in a single test.

3.4.5 Sequencing of Hematological Malignancies

3.4.5.1 Myelodysplastic Syndromes

Myelodysplastic syndromes (MDSs) are clinically heterogeneous disorders characterized by clonal hematopoiesis, impaired differentiation, peripheral blood cytopenias, and a risk of progression to acute myeloid leukemia (AML). Somatic mutations may influence the clinical phenotype but are not included in current prognostic scoring systems. Combination of genomic approaches, including NGS and mass spectrometry-based genotyping, identified somatic mutations in 18 genes in samples of bone marrow aspirate from patients with MDS and associated them with specific clinical features (Bejar et al. 2011). Mutations in TP53, EZH2, ETV6, RUNX1, and ASXL1 were found to be predictors of poor overall survival in MDS patients independently of established risk factors.

The genetic changes that underlie progression from the MDS to secondary AML are not well understood. Whole-genome deep sequencing using Illumina GAIIx or HiSeq was performed on paired samples of skin and bone marrow from subjects with secondary AML to identify somatic mutations specific to secondary AML (Walter et al. 2012). Nearly all the bone marrow cells in patients with MDS and

secondary AML were found to be clonally derived. Thus, genetic evolution of secondary AML is a dynamic process shaped by multiple cycles of mutation acquisition and clonal selection. These results clearly establish that MDS is truly an early form of cancer. The results also suggest that it may be possible to get a therapeutic advantage in AML by targeting the mutations present in the original clonal population. However, further research will be required to find out whether a mutation is in the founding clone that initiated the cancer or in a later-evolving clone.

3.4.5.2 Acute Myeloid Leukemia

The molecular pathogenesis of AML has been studied with the use of cytogenetic analysis for several years. Recurrent chromosomal structural variations are well established as diagnostic and prognostic biomarkers, suggesting that acquired genetic abnormalities (i.e., somatic mutations) have an essential role in pathogenesis. However, the full complement of DNA mutations that are responsible for the pathogenesis of AML is not yet known, but several sequencing studies have been done.

Massively parallel DNA sequencing was used to obtain a very high level of coverage (~98 %) of a primary, cytogenetically normal, de novo genome for AML with minimal maturation and a matched normal skin genome (Mardis et al. 2009). The AML genome that was sequenced contains ~750 point mutations, of which only a small fraction are likely to be relevant to pathogenesis. By comparing the sequences of tumor and skin genomes of a patient with AML-M1, recurring mutations were identified that may be relevant for pathogenesis of AML. However, some studies have shown that many patients with AML carry no mutations in any of the currently recognized driver genes associated with the pathogenesis of AML.

Sequencing has been used to analyze mutations in 18 genes in tumors from individuals with AML, looking at how alterations in these genes are related to survival rates and response to increase in chemotherapy dose (Patel et al. 2012). Results showed that patients whose tumors contained alterations to either DNMT3A or NPM1, survival rates improved when higher-than-usual doses of the chemotherapy drug daunorubicin were used. The same was true for individuals whose tumors harbored MLL gene translocations. Information of this type is useful for a clinician for planning of treatment at time of diagnosis and the start of therapy. If the patient has the mutation in question, the clinician can go ahead and give the higher chemotherapy dose, but if the patient does not have the mutation, a higher dose may not be of benefit.

Given that most bone marrow cells are short-lived, the accumulation of multiple leukemogenic mutations in a single clonal lineage has been difficult to explain. Serial acquisition of mutations has been postulated to occur in self-renewing hematopoietic stem cells (HSCs). Genomic analysis of HSCs from six patients with de novo AML using next-generation exome sequencing has revealed mutations present in individual AML patients harboring the FLT3-ITD (internal tandem duplication) mutation (Jan et al. 2012). In addition to their sequencing approach, the team also used high-throughput flow cytometry to identify biomarkers specific for a patient's healthy HSCs versus their leukemia stem cells and to isolate the very rare populations of precancerous HSCs. The genetic sequences from the precancerous blood

stem cells were then compared to the same regions from the patients' leukemia-plagued stem cells. Analysis revealed the exact order of rare mutations that blood stem cells accrued to become cancerous. Some of these mutations include those in the NPM1, TET2, and SMC1A genes. Finally, through single-cell analysis, a clonal progression of multiple mutations was shown to occur in the HSCs of some AML patients. These preleukemic HSCs suggest the clonal evolution of AML genomes from founder mutations, revealing a potential mechanism contributing to relapse. Such preleukemic HSCs may constitute a cellular reservoir that should be targeted therapeutically for more durable remissions.

The performance of most NGS analysis tools for identifying medium-sized insertions such as FLT3-ITD mutations is largely unknown. A multigene, targeted NGS assay was used to obtain deep sequence coverage of FLT3 and 26 other genes from 22 FLT3 ITD-positive and 29 ITD-negative specimens to examine the performance of several commonly used NGS analysis tools for identifying FLT3-ITD mutations (Spencer et al. 2013). ITD mutations were present in hybridization-capture sequencing data, and Pindel was the only tool out of the seven tested that reliably detected these insertions. Pindel had 100 % sensitivity and 100 % specificity; it provided accurate ITD insertion sizes and was able to detect ITD alleles present at estimated frequencies as low as 1 %. These data demonstrate that FLT3-ITDs can be reliably detected in panel-based, NGS assays.

3.4.5.3 Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL) is a malignancy of the bone marrow, in which there is a deficiency of myeloid cells and an excess of immature cells called promyelocytes. APL is most commonly caused by a translocation (15:17) and expression of the promyelocytic leukemia and the retinoic receptor-α (PML–RARA) fusion product; however, the events that cooperate with PML–RARA in APL pathogenesis are not well understood. An innovative approach has been used to find other relevant mutations in APL. WGS and copy number analysis of a well-characterized APL mouse model has uncovered somatic mutations in Jak1 and lysine (K)-specific demethylase 6A (Kdm6a, also known as Utx) in mice with APL and validated the ability of Jak1 mutations to cooperate with PML–RARA in APL (Rampal and Levine 2011). The findings implicate the JAK/STAT pathway in the pathogenesis of APL and illustrate the power of WGS to identify novel disease alleles in murine models of disease.

3.4.5.4 Chronic Myelomonocytic Leukemia

Ultradeep NGS has been used in hematological malignancies for diagnosis as well as disease classification. It has been applied in chronic myelomonocytic leukemia (CMML), which is a clonal hematopoietic malignancy characterized by features of myeloproliferative neoplasm as well as a MDS, and where data on a comprehensive cytogenetic or molecular genetic characterization are limited. NGS technology was used to investigate CBL, JAK2, MPL, NRAS, and K-ras at known mutational

hotspot regions in CMML (Kohlmann et al. 2010). Cytogenetic aberrations were found in 18.2 % of patients; in contrast, at least one molecular mutation was observed in 72.8 % of patients. NGS screening has been demonstrated to support a comprehensive characterization of the molecular background in CMML. A pattern of molecular mutations translates into different biological and prognostic categories of CMML. An ongoing study, IRON (Interlaboratory Robustness of NGS), is evaluating targeted sequence PCR (Fluidigm, NimbleGen, and RainDance) and PCR cleanup (Agencourt).

3.4.5.5 Hairy Cell Leukemia

Hairy cell leukemia (HCL), a cancer of the bone marrow, is a well-defined clinicopathologic entity whose underlying genetic lesion is still obscure. HCL results in accumulation of abnormal B lymphocytes in the blood. Roughly 2,000 new cases of HCL are diagnosed annually in the USA and Europe. Whole-exome sequencing identified five missense somatic clonal mutations that were confirmed on Sanger sequencing, including a heterozygous mutation in BRAF that results in the BRAF V600E variant protein (Tiacci et al. 2011). Since BRAF V600E is oncogenic in other tumors, further analyses were focused on this genetic lesion. None of the patients with other peripheral B-cell lymphomas or leukemias who were evaluated carried the BRAF V600E variant. It was concluded that BRAF V600E mutation is present in all patients with HCL, and this finding may have implications for the pathogenesis, diagnosis, and targeted therapy of HCL. It also provides an immediate therapeutic indication for the use of available anti-B-raf drugs. Trovagene has licensed this technology for diagnostic applications, and assay may help physicians monitor the effectiveness of treatment and disease relapse.

3.4.5.6 Sequencing in Chronic Neutrophilic Leukemia and Atypical CML

Chronic neutrophilic leukemia (CNL) and atypical (BCR–ABL1-negative) chronic myeloid leukemia (CML) are diagnosed on the basis of neoplastic expansion of granulocytic cells and exclusion of genetic drivers that are known to occur in other myeloproliferative neoplasms. An integrated approach of deep sequencing coupled with the screening of primary leukemia cells obtained from patients with CNL or atypical CML against panels of tyrosine kinase-specific siRNAs or small-molecule kinase inhibitors has been used to identify potential genetic drivers in these disorders (Maxson et al. 2013). The investigators validated candidate oncogenes using in vitro transformation assays and drug sensitivities with the use of assays of primary-cell colonies. Activating mutations were identified in the gene encoding the receptor for colony-stimulating factor 3 (CSF3R) in 59 % of patients with CNL or atypical CML. These mutations segregate within two distinct regions of CSF3R and lead to preferential downstream kinase signaling through SRC family-TNK2 or JAK kinases and differential sensitivity to kinase inhibitors. A patient with CNL carrying a JAK-activating CSF3R mutation had marked clinical improvement after the

administration of the JAK1/2 inhibitor ruxolitinib. It was concluded that mutations in CSF3R are common in patients with CNL or atypical CML and represent a potentially useful criterion for diagnosing these neoplasms.

3.4.6 Sequencing of Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC), one of the most common virus-associated cancers, is the third most frequent cause of cancer-related death worldwide. Massively parallel sequencing of a primary hepatitis C virus (HCV)-positive HCC and matched lymphocytes from the same individual using Illumina GAIIx has led to identification of >11,000 somatic substitutions of the tumor genome that showed predominance of T>C/A>G transition and a decrease of the T>C substitution on the transcribed strand, suggesting preferential DNA repair (Totoki et al. 2011). The authors further validated previously uncharacterized mutation patterns, intrachromosomal rearrangements, and fusion genes, as well as genetic heterogeneity within the tumor. Whole-exome sequencing at a high sequence depth revealed a TSC1 nonsense substitution in a subpopulation of the tumor cells. This first high-resolution characterization of a virus-associated cancer genome identified previously uncharacterized mutation patterns, intrachromosomal rearrangements, and fusion genes, as well as genetic heterogeneity within the tumor. Of the 670 insertion and deletion (indel) patterns detected in the tumor, 7 affected genes; 2 of these changes altered known tumor suppressors, TP53 and AXIN1, while 5 were in genes linked to other types of cancer. The findings included alterations in TSC1, a gene that codes for a component of a protein complex, which is inactivated in a subpopulation of tumors and negatively regulates the mammalian target of rapamycin signaling—an important oncogenic pathway related to the growth, metabolism, and stemness of cancer cells. This could be a promising molecular therapeutic target in HCC progression.

Another genome-wide association study using Illumina HumanHap610-Quad and HumanHap550v3 Genotyping BeadChip in 721 individuals with HCV-induced HCC and HCV-negative controls of Japanese origin detected eight SNPs that showed possible association (Kumar et al. 2011). Subsequent analyses using individuals with chronic hepatitis C (CHC) revealed a lone risk locus near the chromosome 6 gene MICA that is significantly associated with progression from CHC to HCC. Although the molecular mechanism whereby MICA polymorphisms confer the risk of disease progression needs to be characterized in the future, these findings reveal a crucial role of genetic variations in host innate immune system in the development of HCV-induced HCC.

3.4.7 Sequencing of Melanoma

Earlier genome sequencing study of malignant melanoma has shown that skin cancers tend to have a particularly high DNA mutation rate, partly reflecting the

environmental component of these cancers, since UV light can cause extensive DNA damage. Some genes have been linked to the disease already, notably the BRAF gene, which can be targeted therapeutically. However, much remains unknown about the repertoire of mutations that can underlie melanoma. Recent sequencing of 14 melanoma exomes along with the exomes for matched normal blood samples led to identification of previously unrecognized mutations affecting nearly 70 genes including a recurrent mutation in TRRAP in 4 % of cases as well as mutations in GRIN2A in 33 % of tumors (Wei et al. 2011). These findings point to a role for glutamate signaling processes in skin cancer. Although the glutamate pathway has been tied to melanoma and other types of cancer before, this study marks the first time certain genes within this pathway have been linked to melanoma. To achieve this, the researchers captured 37 million bases of coding sequence—representing ~20,000 genes—with the Agilent SureSelect system for each tumor and matched normal sample. They then sequenced the tumor and normal exomes to a mean depth of 180 times or more using the Illumina GAII. Even after filtering variants in tumor exomes against those in matched normal exomes, the dbSNP database, and 1,000 Genomes Project data, they were still left with nearly 5,200 apparent somatic mutations affecting 3,568 genes in the tumors. By doing targeted Sanger sequencing, incorporating information on sequence coverage, gene size, and extent of mutation in affected genes, and applying a so-called MPG algorithm, the team was able to narrow in on 68 genes containing somatic changes. Of these, 16 genes are suspected to contain driver mutations. Whole-exome sequencing is continuing on additional melanoma samples. The researchers also hope to compare the mutations found in primary tumors with those in metastatic samples from the same individual to get a better idea of which mutations occur earliest and to learn more about whether mutations are shared within different metastases. The next challenge is to determine which of these alterations are the most important and using that information for patient care. Functional studies are needed to determine the consequences of driver mutations and more.

Synonymous mutations affect protein function, but they are rarely investigated in oncogenomics. A study used whole-genome and whole-exome sequencing to identify somatic mutations in melanoma samples and validated a synonymous somatic mutation in bcl2L12 that harbored the recurrent F17F mutation (Gartner et al. 2013). Protein made from mutant bcl2L12 transcript bound p53, inhibited UV-induced apoptosis more efficiently than wild-type bcl2L12, and reduced endogenous p53 target gene transcription. The data indicate that silent alterations have a role to play in human cancer and emphasize the importance of their investigation in future cancer genome studies.

3.4.8 Sequencing of Ovarian Cancer

A catalog of molecular aberrations that cause ovarian cancer is critical for developing and deploying therapies that will improve patients' lives. TCGA project has

analyzed mRNA expression, miRNA expression, promoter methylation, and DNA copy number in high-grade serous ovarian adenocarcinomas and the DNA sequences of exons from coding genes in most of these tumors (The Cancer Genome Atlas Research Network 2011). The equipment used included Agilent, Illumina, and Affymetrix arrays to look at CNV, mRNA expression, miRNA expression, and methylation profiles of tumor samples. Whole-exome sequencing was carried out on a subset of these. High-grade serous ovarian cancer was found to be characterized by TP53 mutations in almost all tumors. Pathway analyses suggested that homologous recombination was defective in about half of the tumors analyzed and that notch and FOXM1 signaling are involved in serous ovarian cancer pathophysiology. Although relatively few genes were found to contain recurrent mutations in the ovarian cancer, the researchers tracked down numerous CNVs and several frequently mutated pathways, along with miRNA, methylation, and transcription signatures that hold promise for categorizing ovarian cancer and predicting survival outcomes. Overall, these discoveries set the stage for approaches to the treatment of high-grade serous ovarian cancer in which aberrant genes or networks are detected and targeted with therapies selected to be effective against these specific aberrations.

3.4.9 Sequencing of Prostate Cancer

Prostate cancer is the second most common cancer in men worldwide and causes over 250,000 deaths each year. Overtreatment of indolent disease also results in significant morbidity. The full range of prostate cancer genomic alterations is incompletely characterized. Common genetic alterations in prostate cancer include losses of NKX3.1 (8p21) and PTEN (10q23), gains of AR (the androgen receptor gene), and fusion of ETS family transcription factor genes with androgen-responsive promoters.

The complete sequence of seven primary human prostate cancers and their paired normal counterparts, using the Illumina GAII to sequence primary tumor samples, has been published (Berger et al. 2011). Several tumors contained complex chains of balanced, i.e., copy-neutral rearrangements that occurred within or adjacent to known cancer genes. Rearrangement breakpoints were enriched near open chromatin, AR, and ERG DNA binding sites in the setting of the ETS gene fusion TMPRSS2–ERG, a key feature of prostate cancer, but inversely correlated with these regions in tumors lacking ETS fusions. This observation suggests a link between chromatin or transcriptional regulation and the genesis of genomic aberrations. Three tumors contained rearrangements that disrupted CADM2 and four harbored events disrupting either PTEN (unbalanced events), a prostate tumor suppressor, or MAGI2 (balanced events), a PTEN interacting protein not previously implicated in prostate tumorigenesis. Thus, genomic rearrangements may arise from transcriptional or chromatin aberrancies and engage prostate tumorigenic mechanisms. This study emphasizes the advantage of WGS for a disease like prostate cancer, where the rearrangements seem to be important for the disease progression.

When the researchers incorporated epigenetic information into their analysis, they found clues that tumors carrying the TMPRSS2–ERG gene fusion also tend to have more breakpoints in the regions of the genome reported to have open chromatin and active transcription in prostate cancer studies. On the other hand, tumors lacking this fusion have more breakpoints in parts of the genome with closed chromatin regions that are typically transcriptionally silent. More sequencing work is needed to determine whether these patterns hold in additional prostate tumors. If genomes of all prostate cancer risk categories are sequenced, one may be able to distinguish indolent from aggressive prostate cancer based on the sequence information and potentially prevent unnecessary prostatectomies and overtreatment of prostate cancer.

3.4.9.1 Identification of Mutations in Prostate Cancer by Exome Sequencing

Characterization of the prostate cancer transcriptome and genome has identified chromosomal rearrangements and CNVs, including ETS gene family fusions, PTEN loss, and AR amplification, which drive prostate cancer development and progression to lethal, metastatic castration-resistant prostate cancer (CRPC). Sequencing of the exomes of lethal, heavily pretreated metastatic CRPCs obtained at rapid autopsy and treatment-naive, high-grade localized prostate cancers has identified low overall mutation rates even in heavily treated CRPCs and confirmed the monoclonal origin of lethal CRPC (Grasso et al. 2012). They also carried out aCGH and gene expression analyses on both CRPC and localized tumors, as well as matched benign tumor tissue, using Oncomine tool (Compendia Bioscience) to process and analyze their data. Although the overall mutation rate was relatively low in the tumors, including those exposed to extensive treatment, researchers did find nine genes with recurrent somatic mutations; six of the genes had been implicated in the disease before, while 3—MLL2, OR5L1, and CDK12—were new. Consistent with past results, their analysis suggested that PTEN and WNT signaling pathways are often altered in prostate cancer. Potential driver mutations also turned up in known tumor suppressor genes and oncogenes, as well as genes from pathways involved in cell checkpoints, DNA damage repair, and AR signaling. The exomes also contained mutations to genes from histone or chromatin modification-related pathways, e.g., one of the newly discovered genes, MLL2, codes for a histone methyltransferase enzyme belonging to a protein complex that interacts with the AR. That analysis also helped in uncovering a prostate cancer subtype characterized by mutations to the chromatin-modifying enzyme gene CHD1, and subsequent experiments indicated that most tumors containing CHD1 alterations do not harbor ETS gene family fusions. Through a series of follow-up experiments, the team explored the biological basis for some of the mutations detected, looking, for instance, at physical interactions between the AR and the gene products of mutated genes, including FOXA1. In summary, the mutational landscape of heavily treated metastatic cancer identified novel mechanisms of AR signaling deregulation in prostate cancer. The study provides insights into the resistance mechanisms that evolve in refractory tumors.

Recurrent somatic base-pair substitutions are believed to be less contributory in prostate tumorigenesis but have not been systematically analyzed in large cohorts. By sequencing the exomes of prostate tumor and normal tissue pairs with SureSelect system (Agilent Technologies) and Illumina HiSeq 2000, new recurrent mutations were identified in multiple genes, including MED12 and FOXA1 (Barbieri et al. 2012). SPOP was the most frequently mutated gene, with mutations involving the SPOP substrate-binding cleft in 6–15 % of tumors across multiple independent cohorts. Prostate cancers with mutant SPOP lacked ETS family gene rearrangements and showed a distinct pattern of genomic alterations. Thus, SPOP mutations may define a new molecular subtype of prostate cancer. Because of the SPOP gene product's role in helping to mark proteins for destruction through ubiquitin-mediated pathways, researchers hypothesized that mutations to the gene might contribute to cancer development or progression through deregulation of yet unidentified cellular processes. There might be an accumulation of proteins in the cell that are not cleaned out, and this might lead to cancer growth, or the mutations could be removing proteins that help prevent unchecked cell growth. Those involved in the study noted that additional studies will be needed to explore the genetic, epigenetic, and transcriptional interplay within this and other prostate cancer subtypes and to determine whether the presence of SPOP alterations correspond to any discernible prognostic or treatment outcome patterns.

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Chapter 4
Oncoproteomics

4.1 Introduction

The term "proteomics" indicates PROTEins expressed by a genOME and is the systematic analysis of protein profiles of tissues. The term "proteome" refers to all proteins produced by a species, much as the genome is the entire set of genes. Unlike the genome, the proteome varies with time and is defined as "the proteins present in one sample (tissue, organism, cell culture) at a certain point in time." Proteomics parallels the related field of genomics. Now that the human genome has been sequenced, we face the greater challenge of making use of this information for improving healthcare and discovering new drugs. There is an increasing interest in proteomic technologies now because deoxyribonucleic acid (DNA) sequence information provides only a static snapshot of the various ways in which the cell might use its proteins, whereas the life of the cell is a dynamic process. With this background, DNA/RNA (ribonucleic acid) sequences, per se, are not enough for the clear identification of a therapeutic target because proteins and not DNA/RNA are the basis of mode of action of drugs.

The role of proteomics in the study of cancer pathology, cancer diagnostics, and anticancer drug discovery is referred to as oncoproteomics (Jain 2002b, 2008). Relation of oncoproteomics to other technologies is shown in Fig. 4.1. Currently, cancer research is one of the most popular applications of proteomics.

Cancer is a proteomic disease driven by defective protein pathways. Biomarkers are released from tissue microenvironments of cancer, and the entire signaling pathways are the new targets of therapy. Reliable new technologies now exist for multiplex mapping of tissue and cellular phosphoprotein signaling pathways. Phosphoproteins provide a record of ongoing kinases and linear substrate activity in key signaling pathways driven by the pathological state. Study of relevant proteins is useful for gaining an understanding of the pathology and progression of cancer. Proteomic-based approaches, which enable the quantitative investigation of both cellular protein expression levels and protein–protein interactions involved in

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_4,
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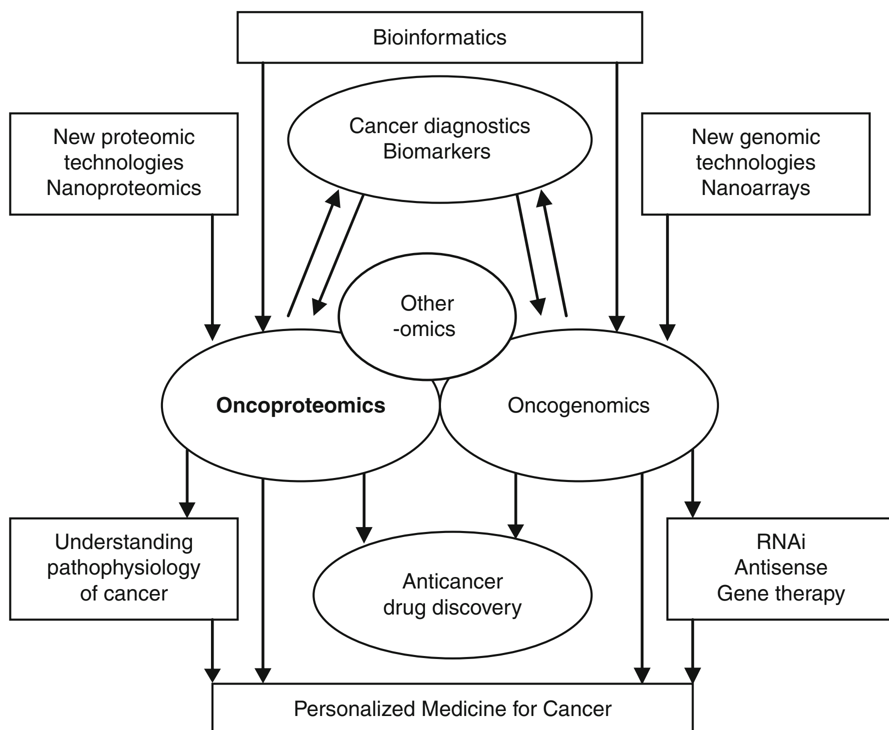

Fig. 4.1 Relation of oncoproteomics to other technologies. © Jain PharmaBiotech

signaling networks, promise to define the molecules controlling the processes involved in cancer. Proteomic-based profiling uniquely allows delineation of global changes in protein expression patterns resulting from transcriptional and posttranscriptional control, posttranslational modifications, and shifts in proteins between different cellular compartments. Given that comprehensive expression profiles obtained using genomics and proteomics are highly complementary, a combined approach to profiling may well uncover expression patterns that could not be predicted using a single approach.

Morphoproteomics combines histopathology, molecular biology, and proteomics to depict the protein circuitry in diseased cells for uncovering molecular targets amenable to specific intervention, thereby facilitating personalized therapy. Such an approach can uncover or confirm potential molecular targets that may be essential to the growth, integrity, and histogenesis of a particular tumor type and that are amenable to specific therapeutic interventions. Directions for future research should focus on points of convergence in signal transduction pathways and consider integration of morphoproteomic with genomic and pharmacoproteomic as well as protein-function microarray data.

4.2 Proteomic Technologies for the Study of Cancer

Several proteomic technologies used for the study of cancer have been described in a detailed report on proteomics (Jain 2013). Some of these are briefly described in this chapter.

4.2.1 Application of CellCarta Technology for Oncology

CellCarta™ (Caprion) enables hypothesis-free studies whereby the proteome of a large number of samples is profiled in order to identify proteins whose abundance differs between study groups or cohorts. Endogenous peptides obtained from digestion with trypsin are first analyzed in scanning mode by mass spectrometry (MS) coupled to liquid chromatography (LC–MS). CellCarta has been applied in areas of special pharmaceutical interest for tumor antigen discovery, protein biomarker discovery, pharmacoproteomics, and protein phosphorylation. Applications in oncology are the most extensive. Isolation of purified membrane proteins from resected tumors is followed by differential analysis of protein abundance. High-value protein targets are identified. Examples of applications in oncology are in lung and colon cancer.

4.2.2 Accentuation of Differentially Expressed Proteins Using Phage Technology

A method called ADEPPT (accentuation of differentially expressed proteins using phage technology) can identify proteins that are produced in different amounts in diseased tissue compared with healthy tissue (Suber et al. 2004). The method uses a large "library" with thousands of strains of the bacteriophage known each making a different peptide that binds to a specific protein. By using a large bacteriophage library, the researchers can detect proteins that are produced at varying levels in different tissue samples. The technique can rapidly determine differences between lung cancer tissue and normal lung tissue by measuring subtle variations in the proteins they produce. ADEPPT can pick up proteins in lung cancer that are overlooked by more conventional methods of protein profiling. This method might enable researchers to detect proteins responsible for all types of cancer and potentially assist them in finding better drug targets to treat various diseases. This information could also be used for early detection of cancers, e.g., by testing for elevated levels of proteins in the blood, which could improve the chances of successful treatment. ADEPPT method may complement existing methods such as 2D gel electrophoresis (GE) by detecting less abundant proteins.

4.2.3 Cancer Tissue Proteomics

Cancer tissue proteomics implies direct tissue profiling and use of imaging MALDI MS to provide a molecular assessment of numerous expressed proteins within a tissue sample. Analysis of thin tissue sections results in the visualization of 500–1,000 individual protein signals in the molecular weight range from 2,000 to >200,000. Laser capture microdissection (LCM), in combination with MS, enables acquisition of protein signatures from a single cell type within a heterogeneous sample. These signals directly correlate with protein distribution within a specific region of the tissue sample. The systematic investigation of the section allows the construction of ion density maps, or specific molecular images, for virtually every signal detected in the analysis. Application of this approach to study proteomics of specific tumors will be described later in this chapter.

Matrix-assisted laser desorption/time-of-flight (MALDI-TOF) MS can be used to generate protein spectra directly from frozen tissue sections from surgically resected cancer specimens. Profiling MALDI MS has been used to monitor alterations in protein expression associated with tumor progression and metastases. Current data suggests that MALDI MS will be superior to immunohistochemical stains and electron microscopy in identifying the site of origin for tumors currently labeled as "tumor of unknown primary." Another application in surgical pathology would be the rapid evaluation of margins of surgical excision of a tumor. Routine analysis of surgical margins by frozen section is very difficult because some cancers invade in a single-cell fashion without producing a grossly identifiable mass. Sensitivity of MS enables detection of even a few tumor cells within a significantly larger portion of tissue.

The capability of MALDI MS to measure susceptibility and response to therapeutic agents in tumor and surrounding tissues is particularly useful in personalized management of cancer. The original protein profile obtained from the primary tumor can be used to influence the selection of therapeutic agents. Levels of chemotherapeutic agents can be measured directly from a tissue biopsy to assess adequacy of delivery to a particular organ site. It will also help in detecting alterations in specific molecular pathways directly modulated or indirectly affected by the anticancer agent. Finally, it could be used to monitor chemotherapy effects on the tumor.

4.2.4 Desorption Electrospray Ionization for Cancer Diagnosis

A modified MS technique, desorption electrospray ionization (DESI), involves aiming a fine water mist at a surface with a pencil-sized tube that also sucks up the fluid after the droplets have mixed with the material in the sample. Whereas ordinary MS is both time- and labor-intensive, DESI not only is portable, but it can also determine the chemical composition of an unprepared sample, e.g., cancer in liver tissue, within 5 s and tell the difference between diseased and non-diseased regions of tissue samples. Another advantage of DESI is that it can detect lipid biomarkers,

whereas conventional MS is good at detection of protein biomarkers. Cancerous regions possess higher levels of certain lipid molecules, which could indicate a significant relationship between lipids and tumor proliferation. The devices might one day prove useful in helping surgeons ensure that all of the tumor is destroyed before a patient leaves surgery and also to identify other potential tumor sites in the tissue that are indistinguishable to the naked eye. It would help physicians determine how well a drug is working in different organs of the body. Analysis of different regions in a tissue sample would facilitate evaluation of the mechanism of its drug action and its effectiveness.

4.2.5 Id Proteins as Targets for Cancer Therapy

Id (inhibitor of DNA binding) proteins represent attractive targets for cancer therapy. Their involvement in the progression of human cancer is now established, based on the analysis of tumor cell cultures, human cancer biopsies, and animal tumor models. Four members of the Id protein family, Id1 to Id4, which lack the basic DNA-binding domain, function as dominant inhibitors of cell cycle regulators. Id proteins within invasive and progressive cancers have distinctive expression patterns that are potential specific diagnostic and/or prognostic biomarkers. Overexpression of Id proteins promotes cancer cell proliferation and resistance against apoptosis.

Multiple strategies can now be used to target the functional activities of intracellular proteins for cancer therapy. These include antisense oligonucleotides, siRNA, anti-gene or ribozyme gene transfer, small molecules, or peptides. However, each of these strategies has potential advantages and disadvantages. Even though numerous genes that are regulated by Id gene expression in cancer cells have been identified, much work must be done to link these associated genes to downstream functional activities.

4.2.6 Identification of Oncogenic Tyrosine Kinases Using Phosphoproteomics

PhosphoScan® proteomics (Cell Signaling Technology Inc.) is used for broad profiling of phosphorylation in cells and tissues. PhosphoScan® profiling involves immunoaffinity purification and tandem MS, and its effectiveness in kinase target and biomarker discovery has been documented. This phosphoproteomic approach has been used to characterize tyrosine kinase signaling across NSCLC cell lines as well as tumors (Rikova et al. 2007). Profiles of phosphotyrosine signaling are generated and analyzed to identify known oncogenic kinases such as epithelial growth factor receptor (EGFR) and c-Met as well as novel anaplastic lymphoma kinase (ALK)

and ROS fusion proteins. Other activated tyrosine kinases such as PDGFRα and DDR1 not previously implicated in the genesis of NSCLC are also identified. By focusing on activated cell circuitry, this approach provides insight into cancer biology not available at the chromosomal and transcriptional levels and can be applied broadly across all human cancers. Further use of this technology has shown that in drug-sensitive cells the targeted tyrosine kinase drives other RTKs and an extensive network of downstream signaling that collapse with drug treatment (Guo et al. 2008). Comparison of the signaling networks in EGFR and c-Met-dependent cells has identified a core network of approximately 50 proteins that participate in pathways mediating drug response.

4.2.7 Laser Capture Microdissection Technology and Cancer Proteomics

LCM technology integrates a standard laboratory microscope with a low-energy laser and a transfer film in a convenient, one-step aim-and-shoot method. LCM provides research or pathology laboratories with the ideal microdissection method and can be combined with proteomic technologies (Jain 2002a). LCM was conceived and first developed as a prototype research tool at the NCI. The commercial version, Arcturus CellPix II Laser Capture Microdissection, is configured to isolate specific cells of interest from microscopic regions of tissue/cells/organisms for later molecular analysis. It offers a sterile method of extracting pure subsets of cells from tissue specimens and cytological smears. Tissue biopsy is examined under a microscope; the cells of interest are located and activated by the laser beam and adhere to the transfer film. The film is placed directly into the DNA, RNA, or enzyme buffer. The cellular material detaches from the film and is ready for subsequent molecular analysis. The tissue remaining on the slide is fully accessible for comparative molecular analysis of adjacent cells. Most importantly, the exact morphologies of both the captured cells and the surrounding tissue are preserved. LCM has been combined with a protein chip and enabling researchers to rapidly generate and analyze protein profiles or fingerprints using relatively small number of cells. Thus, the global changes between normal and disease samples can be readily evaluated.

The fluctuations of expressed genes or alterations in the cellular DNA that correlate with a particular disease stage can ultimately be compared within or between individual patients. Such a fingerprint of gene expression patterns can provide crucial clues for the etiology and might ultimately contribute to diagnostic decision and therapies tailored to individual patients. Molecules found to be associated with a defined pathological lesion might serve as imaging or therapeutic agents.

LCM has been extensively used in cancer research, contributing to the understanding of tumor biology by mutation detection, clonality analysis, epigenetic alteration assessment, gene expression profiling, and proteomics (Cheng et al. 2013). LCM is being used in the Cancer Genome Anatomy Program to catalog the

development of cells from normal to diseased state. It can be applied to any disease process, which is accessible through tissue sampling, such as premalignant cancer lesions. One limitation of LCM is poor quality of nucleic acids and proteins from the archival samples and the drawback of working with frozen samples. To overcome these problems, a rapid simple method for the enrichment of normal and tumor cells was developed, called epithelial aggregate separation and isolation.

4.2.8 Mass Spectrometry for Identification of Oncogenic Chimeric Proteins

The ALK on 2p23 is a tyrosine kinase that forms chimeric fusions with numerous translocation partners. An MS-based approach has been described for the identification of ALK fusion partners (Elenitoba-Johnson et al. 2006). This approach accurately identified the nucleophosmin (NPM)–ALK fusion protein in an anaplastic large-cell lymphoma-derived cell line carrying the t(2;5)(p23;q35) and the TPM3–ALK in a clinical biopsy of inflammatory myofibroblastic tumor carrying the t(1;2)(q21;p23). This study shows the ability of MS to identify oncogenic chimeric proteins resulting from chromosomal rearrangements. This proteomic strategy is readily applicable to the identification of the participating members of any fusion protein encoded from chromosomal translocation, wherein one of the partners is known and suitable antibodies are available. When immunoprecipitation and Western blotting using an antibody against a known translocation partner yield a protein band with a molecular weight distinct from that of the known protein; this band shift from its expected size raises the possibility of the presence of a chimeric fusion protein. This will enable the rapid identification of protein partners encoded by chromosomal translocations and represents a potential opportunity for a discovery method for the identification of fusion proteins in malignant neoplasms.

Several approaches to enrich for tyrosine phosphoproteins in cancer cells for subsequent LC–tandem MS analysis using lysates from SU-DHL-1 cells, which express the NPM–ALK as a model system, have been evaluated (Schumacher et al. 2007). Cells were grown in the presence or absence of the phosphatase inhibitor sodium orthovanadate, and tyrosine phosphoproteins were subsequently enriched by immunoprecipitation or immunoaffinity chromatography and protein identification performed by LC–tandem MS. Results show that sodium orthovanadate improves enrichment and thus detection of tyrosine phosphoproteins. Immunoprecipitation of tyrosine phosphoproteins using two different antiphosphotyrosine antibodies increased the number of protein identifications. Finally, peptides from proteins enriched by immunoprecipitation are more abundant than those enriched by immunoaffinity chromatography, and relatively few proteins were found in common. These data demonstrate the utility of an enrichment strategy for the MS-based identification of tyrosine phosphoproteins and show the advantage of complementary techniques for greater protein identification.

4.2.9 Proteomic Analysis of Cancer Cell Mitochondria

A combination of reverse-phase protein microarray with radiolabeled glucose metabolic studies showed that there is a specific association between altered cytochrome c oxidase subunit levels and altered metabolism in cancer cells (Krieg et al. 2004). Mutations in mitochondrial DNA have been frequently reported in cancer cells. Significance of gene expression patterns is not established yet. The role of proteomics in the study of mitochondrial proteome in cancer is as follows:

- Identification of abnormally expressed mitochondrial proteins in cancer cells is possible by mitochondrial functional proteomics.
- Proteomics can identify new markers for early detection and risk assessment, as well as targets for therapeutic intervention.

4.2.10 ProteinChip System

The ProteinChip System (Bio-Rad) is an extremely rapid and powerful proteomic tool for highlighting the differences in protein expression profiles directly from complex tissue lysates and therefore will have a high impact in cancer.

4.2.11 Proteomic Study of p53

The p53 protein is thus one of the main lines of defense against cancer. Almost half of all cancers involve a mutation of the gene for p53. The p53 transcription factor is found in every cell of the body, where it helps to prevent cancer by activating and deactivating the right genes. When the cell is exposed to potentially carcinogenic stress, such as DNA damage or oxygen deficiency, p53 can switch on the genetic program for cell death, preventing the cancer from spreading to the rest of the body. p53 triggers cell cycle arrest and apoptosis through transcriptional regulation of specific target genes. The effect of p53 activation on the proteome has been investigated using 2D GE analysis of mitomycin C-treated HCT116 colon carcinoma cells carrying wild-type p53 (Rahman-Roblick et al. 2007). Of the numerous protein spots separated in overlapping narrow-pH-range gel strips, 115 showed significant expression changes upon p53 activation. The identity of 55 protein spots was obtained by MS, and most of these were not known to be connected to p53 previously. The proteins fall into different functional categories, such as mRNA processing, translation, redox regulation, and apoptosis, consistent with the idea that p53 regulates multiple cellular pathways. p53-dependent regulation of five of the upregulated proteins, eIF5A, hnRNP C1/C2, hnRNP K, lamin A/C, and Nm23-H1, and two of the downregulated proteins, Prx II and TrpRS, was examined in further detail. Analysis of mRNA expression levels demonstrated both transcription-dependent and transcription-independent regulation among the identified targets. Thus, this

study reveals protein targets of p53 and highlights the role of transcription-independent effects for the p53-induced biological response. Many of the mechanisms were previously unknown, and in several cases, changes are seen only at protein level and not at gene level. This new information will be of value for the development of new therapies.

4.2.12 Role of Proteomics in the Study of Cancer Stem Cell Biology

Embryonic stem cells (ESCs) rely on Polycomb group proteins to reversibly repress genes required for differentiation. Stem cell Polycomb group targets are up to 12-fold more likely to have cancer-specific promoter DNA hypermethylation than nontargets, supporting a stem cell origin of cancer in which reversible gene repression is replaced by permanent silencing, locking the cell into a perpetual state of self-renewal and thereby predisposing to subsequent malignant transformation (Widschwendter et al. 2007). Cancer stem cells (CSCs) are stemlike cells that drive tumor growth and metastasis formation, but little is known about the regulation of CSC maintenance pathways in cancer and how these are affected by cancer-specific genetic alterations and by treatment. Proteomics is emerging as a powerful tool to identify the signaling complexes and pathways that control multi- and pluripotency and CSC differentiation (Kranenburg et al. 2012).

4.2.13 Single-Cell Protein Expression Analysis by Microfluidic Techniques

The analysis of single cells obtained from needle aspirates of tumors is constrained by the need for processing. Microfluidic approaches have been applied to measure the expression of surface proteins in single cancer cells or in small populations. Two approaches involve (1) indirect fluorescence labeling of cell surface proteins and channeling of cells in a microfluidic device past a fluorescence detector for signal quantification and analysis and (2) passing coated channeled cells in a microfluidic device over detection zones with ligands to surface proteins and measuring rates of passage and of retardation based on transient interactions between surface proteins and ligands. Experiments measuring passage retardation showed significant differences in passage rates between FGF-2-treated and FGF-2-untreated MCF-7 cells over reaction regions coated with fibronectin and antibody to integrin α5β1 compared with control regions (Fitzpatrick et al. 2006). Blocking peptides reversed the retardation, demonstrating specificity. Thus, immunofluorescence detection in a microfluidic channel demonstrates the potential for assaying surface protein expression in a few individual cells and will enable the development of future methods that do not require cell handling. The flow retardation device represents the first application of this technology for assessing cell surface protein expression in cancer cells

and may provide a way for analyzing expression profiles of single cells without preanalytic manipulation.

4.2.13.1 Dynamic Cell Proteomics in Response to a Drug

The variability of the protein response of human cancer cells to a chemotherapy drug, camptothecin, has been studied by a dynamic-proteomic approach that measures the levels and locations of nearly 1,000 different endogenously tagged proteins in individual living cells at high temporal resolution (Cohen et al. 2008). All cells show rapid translocation of proteins specific to the drug mechanism, including the drug target (topoisomerase-1), and slower, wide-ranging temporal waves of protein degradation and accumulation. However, the cells differ in the behavior of a subset of proteins. The proteins, whose dynamics differ widely between cells, can be identified in a way that corresponds to the outcomes—cell death or survival. This opens the way to understanding molecular responses to drugs in individual cells.

4.3 Integration of Cancer Genomics and Proteomics

The current trend is to integrate genomics, transcriptomics, and proteomics for the profiling of tumor tissues, an approach referred to as operomics. The major goals are the molecular classification of tumors and the identification of markers for the early detection of cancer. Molecular analyses of tumors rely on microdissected tissues, which are simultaneously investigated for genomic, transcriptomic, and proteomic changes. Genomic alterations in tumor cells being investigated include deletions, amplifications, and methylation changes across the entire genome as well as point mutations in specific genes. Expression analysis at the RNA level is being undertaken using oligonucleotide- and complementary DNA (cDNA)-based microarrays. An important aspect of this approach is the large-scale identification and quantitative analysis of tumor proteins in whole-cell lysates as well as in protein compartments. Protein separation strategies include 2D PAGE and LC. Specific protein subsets of interest include membrane proteins, secreted proteins, and antigenic proteins as sources of biomarkers for early detection of cancer.

4.4 Use of Proteomics in Cancers of Various Organ Systems

4.4.1 Proteomics of Brain Tumors

4.4.1.1 Malignant Glial Tumors

Human brain astrocytomas range from the indolent low-grade to the highly infiltrating and aggressive high-grade form, also known as glioblastoma multiforme. The extensive heterogeneity of astrocytic tumors complicates their pathological classification.

A study has compared the protein pattern of low-grade fibrillary astrocytomas to that of glioblastoma multiforme by 2D GE (Odreman et al. 2005). The level of most proteins remains unchanged between the different grade tumors, and only few differences are reproducibly observable. Fifteen differentially expressed proteins, as well as 70 conserved spots, were identified by MS in this study. Western and immunohistochemical analysis confirmed the differential expression of the identified proteins. These data provide an initial reference map for brain gliomas. The proteins more highly expressed in glioblastoma multiforme included peroxiredoxins 1 and 6, the transcription factor BTF3, and alpha-B-crystallin, whereas protein disulfide isomerase A3, the catalytic subunit of the cAMP-dependent protein kinase, and the glial fibrillary acidic protein are increased in low-grade astrocytomas. These findings contribute to deepening our knowledge of the factors that characterize this class of tumors and, at the same time, can be applied toward the development of novel molecular biomarkers potentially useful for an accurate classification of the grade of astrocytomas.

Screening and evaluation of protein biomarkers for the detection of glioblastomas and their distinction from healthy individuals and benign gliomas have been done by using SELDI-TOF MS coupled with an artificial neural network algorithm (Liu et al. 2005). An accuracy of 95.7 %, sensitivity of 88.9 %, specificity of 100 %, positive predictive value of 90 %, and negative predictive value of 100 % were obtained in a blinded test set comparing glioma patients with healthy individuals; an accuracy of 86.4 %, sensitivity of 88.9 %, specificity of 84.6 %, positive predictive value of 90 %, and negative predictive value of 85.7 % were obtained when patient's gliomas were compared with benign brain tumor. Total accuracy of 85.7 %, accuracy for grade I–II astrocytoma of 86.7 %, accuracy for III–IV astrocytoma of 84.6 % were obtained when grade I–II astrocytoma was compared with grade III–IV ones (discriminant analysis). MALDI MS tissue profiling technology has been used to detect differentially expressed proteins in high-grade and low-grade astrocytomas with level I confidence for prognosis (Holt et al. 2006). SELDI-TOF MS combined with bioinformatic tools could greatly facilitate the discovery of biomarkers with sensitivity and specificity for the discrimination of glioma patients from healthy individuals and those with brain benign tumors.

4.4.1.2 Meningiomas

Meningiomas arise from the coverings of the brain and are usually considered to be benign tumors but may become malignant. They are classified into three groups (benign, atypical, and anaplastic) based on morphologic characteristics. Atypical meningiomas, which are WHO grade 2 tumors, and anaplastic meningiomas, which are WHO grade 3 tumors, exhibit an increased risk of recurrence and premature death compared with benign WHO grade 1 tumors. Although atypical and anaplastic meningiomas account for <10 % of all of meningiomas, it can be difficult to distinguish them from benign meningiomas by morphologic criteria alone.

Proteomic technologies have been applied to improve the diagnosis and grading of meningiomas. Tissue obtained from human meningiomas by selective tissue

microdissection was examined by 2D GE to determine protein expression patterns (Okamoto et al. 2006). Proteomic analysis revealed protein expression patterns unique to WHO grade 1, 2, and 3 meningiomas and identified 24 proteins that distinguish each subtype. Fifteen proteins showed significant changes in expression level between benign and atypical meningiomas, whereas nine distinguished atypical from anaplastic meningiomas. Differential protein expression was confirmed by Western blotting and immunohistochemistry (IHC). The authors of this study established differential proteomic profiles that characterize and distinguish meningiomas of increasing grades. The proteins and proteomic profiles enhance the understanding of the pathogenesis of meningiomas and have implications for diagnosis, prognosis, and treatment.

4.4.1.3 DESI-MS for Intraoperative Diagnosis of Brain Tumors

The goal of brain tumor surgery is to maximize tumor removal while preserving brain function. However, existing imaging and surgical techniques do not offer the molecular information needed to delineate tumor margins. A system has been developed to rapidly analyze and classify brain tumors based on lipid information acquired by DESI-MS to build a classifier to discriminate gliomas and meningiomas (Eberlin et al. 2013). The classifier was tested and results were validated for intraoperative use by analyzing and diagnosing tissue sections from surgical specimens obtained from oligodendroglioma, astrocytoma, and meningioma tumors of different histological grades. The molecular diagnosis derived from MS imaging corresponded to histopathology diagnosis with very few exceptions. This study demonstrates that DESI-MS technology has the potential to guide brain tumor surgery by providing rapid diagnosis, by identifying the histological type and grade of brain tumors, as well as by defining tumor margins in near real time.

4.4.2 Proteomics of Breast Cancer

Proteomic-based approaches are now being used to study the natural history and treatment of breast cancer. 2D PAGE is still the basis of most proteomic studies. Newer technologies such as LCM and highly sensitive MS methods are currently being used together to identify greater numbers of lower-abundance proteins that are differentially expressed between defined cell populations. Proteomics has the refinement and sensitivity to find proteins that are either uniquely or differentially expressed between different cell types, the consequences of which could enable new strategies for drug discovery. The global divergence between normal and tumor breast cells can be identified by using proteomics and can facilitate the development of new approaches to breast cancer treatment and monitoring.

Mammary ductal cells are the origin for 70–80 % of breast cancers. Nipple aspirate fluid (NAF) contains proteins directly secreted by the ductal and lobular

epithelium in non-lactating women. NAF has been used for many years as a potential noninvasive method to identify markers for breast cancer risk or early detection. Scientists at the Tissue Proteomics Unit of the FDA are exploring the use of SELDI-TOF MS to identify patterns of proteins that might define a proteomic signature for breast cancer. They have presented data showing that SELDI analysis of NAF is rapid, reproducible, and capable of identifying protein signatures that appear to differentiate NAF samples from breast cancer patients and healthy controls, including those with an abnormal mammogram who were later proven to be biopsy normal.

Proteomic approaches offer a largely unbiased way to evaluate NAF as a source of biomarkers and are sufficiently sensitive for analysis of small NAF volumes (10–50 μL). There is need for an early detection screening test for breast cancer. Scientists at Power3 Medical have identified 12 blood serum protein biomarkers, whose concentrations differentiate between breast cancer and benign tumors as well as demonstrate statistically significant changes between these groups. They show progressive changes across the disease continuum as well as changes that return to normal at a later date. In addition, they demonstrate changes in disease mechanism measured in the patient's blood.

Human epidermal growth factor receptor-2 (HER-2) gene is amplified in 20–30 % of breast cancers and is associated with response to Herceptin (Genentech) and its lack with resistance to therapy. HER-2/neu status of the primary breast cancer is determined by IHC and fluorescent in situ hybridization (FISH). HercepTest (DakoCytomation), an approved IHC test, is used to identify patients with breast cancer whose tumor tissue overexpresses the HER-2 protein. HER-2 FISH pharmDx™ Kit is designed to quantitatively determine interphase HER-2/neu gene amplification. Mammary5 trial by the National Cancer Institute of Canada showed that amplification of HER-2 in breast cancer cells is associated with better clinical responsiveness to anthracycline-containing chemotherapy regimen when compared with the regimen of cyclophosphamide, methotrexate, and fluorouracil (Pritchard et al. 2006). However, due to technical factors, tests used for primary breast cancer may not accurately reflect the metastatic tumor in terms of HER-2/neu status. Guidelines recommend that tumors should be defined as HER-2/neu positive if 30 % or more of the cells are 3+. Circulating levels of the HER-2 extracellular domain can be measured in serum using an approved test, and increased serum HER-2/neu levels to >15 ng/mL reflect breast cancer progression (Carney et al. 2007).

Protein biomarkers are potentially useful for diagnosis of basal breast tumors, which are particularly difficult to diagnose with mammograms. Similar to these cancers, ER-negative breast cancers are detected through other methods as they often occur in younger women, who tend to have denser breast tissue, which makes mammography less successful. These women could also benefit from an additional blood test to pick up biomarkers. Autoantibodies are promising blood-based markers. Although individual autoantibodies are unlikely to enhance early detection, multiplexed assays for autoantibody panels may achieve the required sensitivity. A technology under development at Arizona State University, NAPPA (Nucleic Acid Programmable Protein Arrays) enables the creation of high-density, customized protein arrays that could be used for this purpose. The idea is to display several

different proteins in a sample, so the autoantibodies in a patient's serum can find any proteins they happen to recognize. The arrays need to be fairly stable and should display proteins that will not change or unfold over time. The NAPPA approach is to take cloned copies of genes and print them on the array. The cloned copies, cDNA, and are configured in such a way that an epitope tag can be added at the C-terminus of the gene. Anything that is captured by virtue of the tag must have the full-length protein attached to it. Those genes are printed on the array, which can then be stored for months. Once the array is needed, it is floated in expression extract that transcribes and translates the proteins in situ on the glass. Those proteins are made about an hour before they are used, guaranteeing that they are as fresh as possible.

The technology uses minimal samples, as little as 0.01 mL of plasma or serum, and has good assay reproducibility and reliability based on pilot work, making it particularly attractive for molecular epidemiology studies. NAPPA technology and other proteomic platforms are another extension of genomic technologies that might provide novel biomarkers for breast cancer. However, this is a new area and the biology of autoantibodies is not well understood. There is need to learn how age and lifestyle factors, as well as intra-person and inter-person variability, influence these biomarkers. Because basal breast cancers are highly aggressive, it may not be possible to isolate autoantibody biomarkers appropriate for early detection, and one may only find biomarkers related to overall tumor aggressiveness. The ultimate goal of the research is to identify candidate autoantibody biomarkers that may detect basal breast cancers early or predict survival.

4.4.2.1 Integration of Proteomic and Genomic Data for Breast Cancer

A study has integrated information from analysis of primary breast cancers on five platforms: (1) genomic DNA copy number arrays, (2) DNA methylation, (3) exome sequencing, (4) mRNA arrays and miRNA sequencing, and (5) reverse-phase protein arrays (Koboldt et al. 2012). The ability to integrate information across platforms, each of which shows significant molecular heterogeneity, provided key insights into previously defined gene expression subtypes and demonstrated the existence of four main breast cancer classes. Somatic mutations in only three genes (TP53, PIK3CA, and GATA3) occurred at >10 % incidence across all breast cancers; however, there were numerous subtype-associated and novel gene mutations including the enrichment of specific mutations in GATA3, PIK3CA, and MAP3K1 with the luminal A subtype. Two novel protein expression-defined subgroups were identified, possibly produced by stromal/microenvironmental elements, and integrated analyses identified specific signaling pathways dominant in each molecular subtype including an HER-2/phosphorylated HER-2/EGFR/phosphorylated EGFR signature within the HER-2-enriched expression subtype. Comparison of basal-like breast tumors with high-grade serous ovarian tumors showed many molecular commonalities, indicating a related etiology and similar therapeutic opportunities. The biological finding of the four main breast cancer subtypes caused by different

subsets of genetic and epigenetic abnormalities raises the hypothesis that much of the clinically observable plasticity and heterogeneity occur within, and not across, these major biological subtypes of breast cancer. This study demonstrates benefits of integrating genomic and proteomic data, particularly phosphoproteomics, which provided information beyond what the gene expression could. Proteomic data suggests the existence of two distinct phosphoproteomic-based subtypes within the larger gene expression-based HER-2 subtype—one exhibiting high HER-2 and HER1 signaling activity and the other exhibiting lower levels of such activity. The other example where the protein made one think was the analysis of PI3 kinase signaling, in which a disconnect was found between the PI3K signaling data obtained via the reverse-phase protein arrays analysis and their PI3K mutation data. A pathway-based analysis of the PI3K signaling pathway revealed that what are believed to be protein and phosphoproteomic signatures of PI3K activation did not correlate with PI3K mutations, but did correlate with the loss of negative regulators of that pathway, like loss of INPP4B or loss of PTEN. Thus, there is a discrepancy between the information from mutation and phosphoproteomics. The challenge now is to figure out which of these many different genetic events or protein signatures are going to be biomarkers of responsiveness to drugs like PI3K or mTOR inhibitors. This work is ongoing and the researchers are currently reanalyzing the genetic data based upon protein and phosphoproteomic endpoints.

4.4.3 Proteomics of Colorectal Cancer

Current screening methods for colorectal cancer (CRC) such as fecal occult blood test and colonoscopy have contributed to early detection and reduction of mortality, but most of the carcinomas are still detected at a late stage when prognosis is poor. Proteomic technologies enable a distinction between the healthy patient and the cancer patient with high sensitivity and specificity and could greatly improve early detection and classification systems for CRC (Habermann et al. 2008). A suitable method for detecting new serum biomarkers of CRC by serum protein profiling using SELDI-TOF MS followed by classification tree pattern analysis has been described (Engwegen et al. 2006). These biomarkers have a potential role in monitoring the disease as well as the treatment. However, there is still a need to identify panels of predictive markers to improve response rates and decrease toxicity with the ultimate aim of tailoring treatment to an individual patient and tumor profile. Application of proteomic technologies for CRC remains to be transferred from the bench to the bedside.

Metastasis is a common phenomenon and the major lethal cause of CRC. Differential proteome analysis of two CRC cell lines was conducted using 2D GE combined with MALDI-TOF MS (Zhao et al. 2007). Obvious differences were detected between the protein profiles of colorectal cell lines with varying potential for metastasis. Overexpression of heat shock protein (HSP) 27 was shown to play an important role in metastasis and progression of CRC.

4.4.4 Proteomics of Esophageal Cancer

Normal squamous epithelium and corresponding tumor cells from patients have been subjected to LCM and studied by 2D PAGE. It is possible to visualize disordered proteins and subsequently determine the identity of selected proteins by high-sensitivity MS microsequencing from microdissected cell populations.

A 2D GE-based technique has been used to identify differentially expressed proteins between esophageal cancer cell lines and immortal cell line (Qi et al. 2008). Fifteen proteins were identified with differences of more than fivefold, comprising the downregulation of annexin A2, histone deacetylase 10 isoform beta and protein disulfide isomerase ER-60 precursor, and the upregulation of heat shock 70-kDa protein 9B precursor, solute carrier family 44 Member 3, heterogeneous nuclear ribonucleoprotein L (hnRNP L), eukaryotic translation initiation factor 4A isoform 2, triosephosphate isomerase 1 (TPI), peroxiredoxin 1 (PRX1), formiminotransferase cyclodeaminase form (FTCD), fibrinogen gamma-A chain precursor, kinesin-like DNA-binding protein, lamin A/C, cyclophilin A (CypA), and transcription factor MTSG1. Expression pattern of annexin A2 was verified by Western blotting and IHC. These protein alterations correlate with esophageal malignant transformation.

4.4.5 Proteomics of Hepatic Cancer

Hepatocellular carcinoma (HCC) has been a major clinical challenge due to low early diagnosis rate and poor prognosis. 2D LC–MS/MS, Western blotting, and IHC staining using tissue microarrays have been used to extensively survey abnormal protein expression associated with HCC in clinical tissues (Li et al. 2012). The proteins upregulated in HCC tissues are involved in regulatory processes, such as the granzyme A-mediated apoptosis pathway (The GzmA pathway). The SET complex, a central component in the GzmA pathway, was significantly upregulated in HCC tissue. These proteomic signatures could help to unveil the underlying mechanisms of hepatocarcinogenesis and may be useful for the discovery of candidate biomarkers.

4.4.6 Proteomics of Leukemia

Proteomics is being used to subclassify leukemia, because cytogenetic analysis is costly and time-consuming. Several useful protein biomarkers have been discovered that can rapidly identify different types of leukemia: alpha-enolase, RhoGDI2, annexin A10, catalase, peroxiredoxin 2, tropomyosin 3, and annexin 1. These are differentially expressed in acute myeloid leukemia (AML) and can be used as biomarkers for disease prognosis and outcome (Lopez-Pedrera et al. 2006). They provide potential new targets for rational pathogenesis-based therapies of AML.

Protein expression patterns obtained by 2D GE have been compared and correlated with clinical features in human B-cell chronic lymphocytic leukemia, which is characterized by broad clinical variability; morphologic examination cannot differentiate between various subtypes. Statistical analysis, however, enables the identification of proteins that clearly discriminate between the patient groups with defined chromosomal characteristics or whose expression levels do correlate with clinical parameters such as patient survival. B-cell chronic lymphocytic leukemia patients with shorter survival times exhibit changed levels of redox enzymes, HSP 27, and protein disulfide isomerase, which may be potentially involved in drug resistance.

Despite enormous therapeutic efforts that range from various cytotoxic agents to allogeneic stem cell transplantation, overall survival of patients with AML remains unsatisfying. There is hope that elucidation of the AML-specific proteome will prompt the discovery of novel therapeutic targets and biomarkers in AML (Czibere et al. 2006). The expression level of distinct proteins of bone marrow mononuclear cells (BMMNCs) in patients with AML has been studied using 2D GE and MALDI-TOF MS before inductive treatments (Tian et al. 2007). Changes in the proteins correlate with prognosis. The proteins of the BMMNC of the patients also change when relapse occurs.

Despite enormous therapeutic efforts that range from various cytotoxic agents to allogeneic stem cell transplantation, overall survival of patients with AML remains unsatisfactory. There is hope that elucidation of the AML-specific proteome will prompt the discovery of novel therapeutic targets and biomarkers in AML, which will enable the development of personalized treatment of AML. One major challenge is limitations in protein detection sensitivity, which presents a problem as many of the regulatory proteins that are pivotal in response to therapy occur in low abundance (Gjertsen and Sjoholt 2008). Ongoing developments in proteomic technologies are expected to open new avenues for personalized molecular therapy of AML and improve efficacy and reduce the toxicity of current treatment.

4.4.7 Proteomics of Lung Cancer

Currently, clinical and pathological staging of lung cancer is suboptimal for achieving the goals of assessing prognosis and selecting therapy. Major progress in understanding the molecular basis of lung cancer has been made due to technical developments in proteomic and genomic analyses and their application to diagnosis and prognosis of lung cancer. Exhaled breath condensate collection is a simple and noninvasive technique, which enables the study of a wide variety of biomarkers including proteins by sampling respiratory tract fluid and may be applied to the detection of lung cancer (Conrad et al. 2008). A panel of four serum proteins (carcinoembryonic antigen, retinol-binding protein, alpha1-antitrypsin, and squamous cell carcinoma antigen) is valuable in suggesting the diagnosis of lung cancer may be useful for detecting individuals at high risk for lung cancer (Patz et al. 2007). If a reliable protein profile of lung cancer can be identified that is associated with poor

prognosis, it may provide potential therapeutic targets. The development of a simple serum test for diagnosis of lung cancer before clinical manifestation is feasible and may simultaneously identify the chemotherapeutic agents to which the tumor is sensitive, enabling personalized treatment (D'Amico 2008).

4.4.8 Proteomics of Pancreatic Cancer

The survival rate of pancreatic cancer patients is the lowest among those with common solid tumors, and early detection is one of the most feasible means of improving outcomes. In conventional practice, the use of CA 19-9 levels and imaging techniques is not optimal for detecting small pancreatic lesions. New experimental approaches, such as quantitative proteomics, have shown great potential for the study of cancer and have opened new opportunities to investigate crucial events underlying pancreatic tumorigenesis and to exploit this knowledge for early detection and better intervention. Isotope-coded affinity tag technology for proteomic analysis of human cancer tissue can be used to identify differentially expressed proteins in pancreatic cancer.

One study has compared plasma proteomes between pancreatic cancer patients and sex- and age-matched healthy controls using surface-enhanced laser desorption/ionization coupled with hybrid quadrupole TOF MS (Honda et al. 2005). A discriminating proteomic pattern was extracted from the data using a support vector machine learning algorithm and was applied to two validation cohorts. A set of four mass peaks most accurately discriminates cancer patients from healthy controls with sensitivity of 97.2 % and specificity of 94.4 %. When combined with CA 19-9, 100 % of pancreatic cancers, including early-stage (stages I and II) tumors, were detected. Although a multi-institutional large-scale study will be necessary to confirm clinical significance, the biomarker set identified in this study may be applicable to using plasma samples to diagnose pancreatic cancer.

4.4.9 Proteomics of Prostate Cancer

Although several non-2D GE platforms—SELDI, ICAT, and array-based technologies—are used for research in proteomics of prostate cancer, 2D GE remains the most powerful method for analysis of cellular protein phenotype in spite of its being labor-intensive and time-consuming. It may potentially reveal gene regulations that cannot be detected on a genetic level (Hellstrom et al. 2007).

Gene expression studies of prostate cancer have shown androgen-regulated genes such as prostate-specific antigen (PSA) and several novel cDNAs. Protein expression profiles of androgen-stimulated prostate cancer cells have been generated by 2D GE. MS analysis of androgen-regulated proteins in these cells has identified the metastasis suppressor gene NDKA/nm23, a finding that may explain a

marked reduction in metastatic potential when these cells express a functional androgen receptor. Quantitation of the number of PSA molecules/cell has been conducted on human prostate tissue cells by coupling of LCM with sensitive quantitative chemiluminescent immunoassays. A proteomic-based approach is useful for developing a more complete picture of the protein profile of prostate needle biopsy specimens, and changes in FLNA(7–15), FKBP4, and PRDX4 expression have been confirmed by immunoblot analyses (Lin et al. 2007). This approach may provide useful molecular targets for diagnosis and treatment of prostate cancer.

Specific populations of normal and malignant epithelium from radical prostatectomy tissue specimens procured by LCM have been analyzed by 2D PAGE. The data demonstrate that 2D PAGE analysis of LCM-derived cells can reliably detect alterations in protein expression associated with prostate cancer and that these differentially expressed proteins are produced in high enough levels which could enable their clinical utility as new targets for therapeutic intervention, serum markers, and/or imaging markers.

The proportion of free and complex PSA in serum is used for differentiating between benign and malignant prostate diseases. To further understand the physiological relationship between PSA in seminal plasma and blood, free prostate-specific antigen (fPSA) and complex prostate-specific antigen (cPSA) have been analyzed in blood and in seminal plasma in young healthy men (Savblom et al. 2005). fPSA in blood, but not cPSA, is associated to PSA in semen (approximately 17 % covariation). In blood, cPSA, but not fPSA, increases with age in healthy men, which may reflect an increasing incidence of prostate disease. A semen-based prostate cancer test, designed to improve the accuracy of the PSA test, has identified a prostate cancer biomarker associated with human carcinoma antigen (HCA). When present along with HCA, the biomarker will form the basis of a new prostate cancer diagnostic test being developed by Proteome Systems.

A major cause of treatment failure for prostate cancer is the development of androgen-independent metastatic disease. Id protein family, a group of basic helix–loop–helix transcription factors, has been shown to be involved in carcinogenesis and a prognostic marker in several types of human cancers. A study has examined the expressions of four Id proteins, Id-1, Id-2, Id-3, and Id-4, in clinical prostate cancer specimens as well as nodular hyperplasia specimens by IHC (Yuen et al. 2006). The results indicate that these Id proteins may play a positive role in the development of prostate cancer. Differential Id protein expressions may be a useful marker for poor prognosis, and Id-4 may be a potential prognostic marker for distant metastasis.

4.5 Proteomics and Tumor Immunology

The combination of proteomic with genomic approaches provides new opportunities for tumor immunology investigation. Microarray analysis has identified important changes in a large number of genes at the molecular level of the differentiation

and maturation of dendritic cells, which are the most potent antigen-presenting cells. Proteomic analysis provides information that could not be obtained at the RNA level, such as the separation of different isoforms and the characterization of posttranslational modifications. Proteomics also enables serological screening of tumor antigens. Proteins that elicit humoral response in cancer are identified by 2D Western blot using sera of cancer patient, followed by MS analysis and database search. The proteome-based approach has enabled the definition of several tumor antigens. The frequent occurrence of autoantibodies to certain of these proteins in different cancers may be useful in cancer screening and diagnosis as well as for immunotherapy.

4.6 Proteomics and Study of Tumor Invasiveness

A proteome-wide analysis of variations in serine hydrolase activity permits the classification of human cancer lines into functional subtypes based on tissue of origin and state of invasiveness. Invasiveness-associated enzymes including urokinase and a secreted serine protease have a recognized role in tumor progression. Invasive cancer cells share discrete proteomic signatures that are more reflective of their biological phenotype than cellular heritage, highlighting that a common set of enzymes may support the progression of tumors from a variety of origins and thus represent attractive targets for the diagnosis and treatment of cancer.

4.7 Role of Proteomics in Studying Drug Resistance in Cancer

One of the problems in chemotherapy of cancer is the development of resistance to anticancer drugs. Mechanisms mediating drug resistance are not well understood. Extensive studies during the last decades have identified several mechanisms through which cells escape the cytotoxic effects of a variety of chemotherapeutic drugs. One type of drug resistance is called multidrug resistance (MDR), because selection with one anticancer drug leads to cross-resistance with a wide range of other drugs. These MDR cells express frequently plasma transport proteins like P-glycoprotein. But cellular resistance to chemotherapy is multifactorial and may be affected by the cell cycle stage and proliferation status, biochemical mechanisms such as detoxification, cellular drug transport, or DNA replication and repair mechanisms. Several laboratory techniques, such as polymerase chain reaction, immunocytochemistry, flow cytometry, blotting, and fluorescent microscopy, have been used for the identification of MDR markers and mechanisms.

Advances in proteomic technologies have enabled the identification of multiple proteins involved in drug-resistant cancers. 2D GE, followed by image analysis and MS, can lead to the identification of proteins differentially expressed between

resistant and sensitive cells. This might lead to the development of various strategies to modify the action of such proteins when their inappropriate structure or expression is contributing to drug-resistant disease. Proteomic technologies have been used to evaluate the drug resistance of melanoma cells toward etoposide, cisplatin, fotemustine, and vindesine.

Mutations in EGFR have been identified as a clinical correlate to objective response to gefitinib in patients with lung cancer, but resistance to gefitinib is a problem. Epithelial membrane protein-1, an adhesion molecule, is a surface biomarker whose expression correlates with acquisition of gefitinib resistance (Jain et al. 2005). Glucose-regulated protein (GRP78), a widely used indicator of the unfolded protein response, is induced in the tumor microenvironment. In vitro studies suggest that GRP78 confers chemoresistance to topoisomerase inhibitors, such as doxorubicin. Combined analyses of multiple proteomic studies of various drug-resistant cancer cell lines have revealed that many mechanisms of resistance likely exist in any given drug-selected cancer cell line and that common mechanisms of resistance may be selected in a spectrum of cancer cell lines (Zhang and Liu 2007). These observations suggest that combination therapies targeting multiple mechanisms to sensitize drug-resistant cancers may be necessary to eradicate cancers in the future.

4.8 Future Prospects of Oncoproteomics

Proteomics is facilitating the integration of diagnostics and therapeutics and fulfilling many of the requirements for personalized therapy of cancer. Several proteomic-based tests for cancer detection, for prognosis, and for guidance of anticancer therapy are expected to become available in the future. The impact of proteomics on cancer will not be limited to the identification of new biomarkers for early detection and new targets. Proteomic technologies will be used to design rational drugs according to the molecular profile of the cancer cell and thus facilitate the development of personalized cancer therapy.

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Chapter 5
Biomarkers of Cancer

5.1 Introduction

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of a physiological as well as a pathological process or pharmacological response to a therapeutic intervention. In case of cancer, biomarkers are referred to as cancer biomarkers. Any specific molecular alteration of a cell on DNA, RNA, metabolite, or protein level can be referred to as a molecular biomarker. In the era of molecular biology, biomarkers usually mean molecular biomarkers and can be divided into three broad categories (Jain 2010):

1. Those that track disease progression over time and correlate with known clinical measures
2. Those that detect the effect of a drug
3. Those that serve as surrogate endpoints in clinical trials

The expression of a distinct gene can enable its identification in a tissue with none of the surrounding cells expressing the specific biomarker. In the past decade, molecular dissection of the cancer by means of mRNA expression profiling enabled detailed classification according to tumor subtypes. The traditional system of tumor node metastases (TNM) has been the main tool for identifying prognostic differences among patients and for guiding the treatment. The TNM system is based on the macroscopic and microscopic morphologic examination of pathological samples. Despite the advantage of uniformity for international communications and studies, there are many limitations of this system as a first-line method for prediction and prognosis of cancer. It is difficult to distinguish related disease subtypes, which have different clinical outcomes. Hence there is a need for more exact molecular biomarkers for use in clinical practice. In recent years the discovery of cancer biomarkers has become a major focus of cancer research. The widespread use of prostate-specific antigen (PSA) in prostate cancer screening has motivated researchers to identify suitable biomarkers for screening different types of cancer. Biomarkers are also useful for diagnosis, monitoring cancer progression, predicting recurrence,

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_5,
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Table 5.1 Desirable characteristics of biomarkers for cancer

	Characteristics				
Purpose	Noninvasive	Low cost	Simple to perform	Accurate[a]	Informative (discriminatory)
Screening	+++	+++	+++	+++	+++
Predisposition	+++	+++	+++	+++	+++
Early detection	++	++	++	+++	+++
Prognosis	+	+	+	++	++
Drug response	+++	++	++	+++	+++
Target for drug	NA	+	NA	+++	NA

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+ = low importance, ++ = medium importance, +++ = high importance, NA = not applicable
[a]Low rate of false-negative results

and assessing efficacy of treatment. The advent of targeted therapies such as imatinib (Novartis' Gleevec), trastuzumab (Roche's Herceptin), and rituximab (Roche's Mabthera), where a causal relationship has been established between drug target and therapy, drives the need for biomarkers for selecting the patients for a given therapy as well as for predicting drug resistance. Role of biomarkers in personalized management of cancer is further discussed in Chap. 16.

5.1.1 The Ideal Biomarker for Cancer

The ideal biomarker for cancer would have applications in determining predisposition, early detection, assessment of prognosis, and drug response. It would be an additional advantage if the biomarker could also serve as a target for drug development. Desirable characteristics of molecular biomarkers for cancer are shown in Table 5.1.

No one test meets all these requirements, but these should be kept in mind for selection of diagnostic tests. There is an urgent need for cancer biomarkers with more accurate diagnostic capability, particularly for early-stage cancer.

5.1.2 Single vs. Multiple Biomarkers of Cancer

Because cancer is a polygenic disease, most diagnostic assessments would probably rely on multiple biomarkers. Although few biomarkers may be needed for testing for predisposition, possibly more would be required for early detection, prognosis prediction, and determination of drug response. Such biomarkers might be qualitative for predisposition tests and early detection of lesions, but quantitative for determining differential gene expression (as may be required for determining prognosis and drug response). If gene expression is not confined to a single library, it should be at least eight times the level of expression in the other library to be statistically significant.

Cancer is a product of the tissue microenvironment. Therefore, interactions between the cancer cells, the surrounding epithelial and stromal cells, vascular channels, the

extracellular matrix, and the immune system are the ultimate determinant of the final pathology. Cell surface antigens and receptors, cell-anchored and secreted enzymes, cytokines, and extracellular matrix molecules are the mode of communication between disease cells and the surrounding microenvironment. The outcome is a complex cascade of molecules available for sampling by the ongoing vascular perfusion. Low molecular weight peptides in the blood may comprise a recording of events in the disease microenvironment. The peptidome signature shed from the microenvironment is a reflection of the microenvironment as a whole. In case of cancer biomarkers, cancer specificity is not derived from proteins secreted exclusively by tumor cells. Mass spectrometry (MS) profiling indicates that a higher level of both specificity and sensitivity might be achieved by measuring the combination of biomarkers emanating from both the diseased cells and the reactive cells in the microenvironment. In this way the peptidome can potentially supplant individual single biomarkers and transcend the issues of tumor and population heterogeneity. Therefore, trend in biomarker discovery is moving away from the ideal single, cancer-specific biomarker such as prostate-specific antigen. Despite decades of effort, most single biomarkers have not reached the level of cancer specificity and sensitivity required for routine clinical use in early detection and screening purposes. A growing confluence of scientific data and results point to combinations of blood-borne markers using MS profiling techniques as well as tissue MS profiling strategies, and multiplexed immunoassay providing more superior results than single markers alone.

Genetic alterations in tumor cells often lead to the emergence of growth-stimulatory autocrine and paracrine signals, involving overexpression of secreted peptide growth factors, cytokines, and hormones. Increased levels of these soluble proteins may be exploited as markers for cancer diagnosis and management or as points of therapeutic intervention. The combination of annotation/protein sequence analysis, transcript profiling, immunohistochemistry (IHC), and immunoassay is a powerful approach for delineating candidate biomarkers with potential clinical significance.

5.2 Types of Cancer Biomarkers

Various types of cancer biomarkers are shown in Table 5.2. Biomarkers specific for various cancers are described later in this chapter.

5.2.1 Biomarkers of Epigenetic Gene Silencing in Cancer

Both genetics and epigenetics regulate gene expression in cancer. Regulation by genetics involves a change in the DNA sequence, whereas epigenetic regulation involves alteration in chromatin structure and methylation of the promoter region. DNA methylation represents an epigenetic means of inheritance without associated DNA sequence alterations. Two major mechanisms that foster epigenetic changes

Table 5.2 Types of cancer biomarkers

Types of cancer biomarkers
Genetic biomarkers
Biomarkers of PTEN tumor suppressor gene status
Gene mutations
Oncogenes
Tumor microvesicles or exosomes
DNA biomarkers
DNA repair biomarkers
Gene amplification
Microsatellite instability
Mitochondrial DNA
Viral DNA
RNA biomarkers
microRNAs (miRNAs)
Protein biomarkers
B7 coregulatory ligands
High motility group protein A2
Raised serum lactate dehydrogenase
YKL-40, a secreted glycoprotein
Metabolic biomarkers
Hypoxia-inducible factor-1
Epigenic biomarkers
DNA methylation
Immunological biomarkers
T-cell and cytokine responses
Biomarkers in cancer stem cells (CSCs)
Cripto-1
Circulating tumor cells as cancer biomarkers
Circulating nucleic acids as potential biomarkers of cancer

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are DNA methylation at cytosine bases within a CpG dinucleotide and histone acetylation. Aberrant CpG island methylation has been associated with changes observed in aging and neoplastic cells. A growing list of genes, including known tumor suppressor genes, has been shown to have aberrant CpG island methylation in cancer. These epigenetic events act as alternatives to mutations and deletions to disrupt tumor suppressor gene function.

Until recently, it was believed that only individual single genes were silenced by methylation. But this is not necessarily the case. Loss of gene expression can occur through long-range epigenetic silencing, with similar implications as loss of heterozygosity (LOH) in cancer. Non-methylated genes that reside in a particular area near methylated genes may also be silenced. Their physical proximity to the methylated genes affects their ability to function. A new method to scan the entire genome in the cancer tissue samples enables widespread changes to be identified in specific parts of the genome. In a study of silencing in colorectal cancer (CRC) using this approach, common repression of the entire 4-Mb band of chromosome 2q.14.2 was found to be associated with global methylation of histone H3 Lys9 (Frigola et al. 2006). DNA hypermethylation within the repressed genomic neighborhood was localized to

three separate enriched CpG island "suburbs," with the largest hypermethylated suburb spanning 1 Mb. Further study will determine if these same regions are switched off in other types of cancers. A fundamental difference between genetic and epigenetic alterations in cancer is the irreversible nature of genetic lesions, whereas epigenetic ones are potentially reversible. Cancer therapies, which can reverse DNA methylation, can restore the cell's normal regulation to prevent or stop the progression of cancer. Drugs that promote DNA demethylation are already in clinical trials. Therefore, detection of biomarkers of methylation is important.

5.2.2 Circulating Tumor Cells as Cancer Biomarkers

Circulating tumor cells (CTCs) are potential prognostic biomarkers of cancer and a reliable mean to predict metastasis development. The presence of CTCs as a prerequisite for development of distant metastasis is known since the nineteenth century. In clinical trials, CTC counts have been used as biomarkers for prognostic stratification and evaluation of disease response during therapy. The number of CTCs could be useful for prognosis in early stage of disease, for identification of patients requiring adjuvant therapy, or during follow-up to detect relapses. Other clinical applications are based on the molecular and genetic characterization of CTCs. These applications have been shown to be valuable in several clinical trials of lung cancer and breast cancer.

5.2.3 Circulating Nucleic Acids as Potential Biomarkers of Cancer

Circulating cell-free DNA (ccf-DNA) levels have been studied in plasma and serum samples as biomarkers of cancer. An elevated level of ccf-DNA has been detected in the circulation of cancer patients in comparison with healthy controls (Kohler et al. 2011). Since ccf-DNA in cancer patients often bears similar genetic and epigenetic features to the related tumor DNA, there is evidence that some of the ccf-DNA originates from tumor tissue. This, and the fact that ccf-DNA can easily be isolated from the circulation and other body fluids of patients, makes it a promising candidate as a noninvasive biomarker of cancer. ccf-DNA may also represent an important source of biomarkers at several steps of carcinogenesis, including early detection of preneoplastic lesions and monitoring of cancer. Moreover, levels of plasma DNA could be tested as a potential intermediate biomarker of the efficacy of intervention. It is possible to develop a simple cost-effective blood test, with high sensitivity and specificity that has potential for screening high-risk individuals, for prognostic purposes and to be used as intermediate endpoints of efficacy in chemoprevention and therapeutic trials (Catarino et al. 2012).

5.2.4 HER3 as Biomarker of Cancer

HER3, a coreceptor of HER2, plays an important and dominant role in the functionality and transformation of HER-mediated pathways. Understanding the role of HER3 in oncogenesis as well as its place as a target for anticancer therapy is an ongoing area of research. Determination of biomarkers for clinical benefit from agents targeting HER3 is an essential component of translating basic science into real-world effective anticancer therapies, with the aim of ensuring the patients most likely to benefit from such treatments can be identified. HER2 and HER3 can be targeted by monoclonal antibodies and the potential for HER3 mRNA levels to predict treatment outcome in ovarian cancer (Amler 2010). An understanding of the value of HER3 mRNA as a biomarker is important for clinical benefit of the HER2–HER3 dimerization inhibitor pertuzumab. In conclusion, HER3 mRNA levels may be a biomarker for active ligand-induced HER2–HER3 signaling, with low HER3 mRNA levels correlated with clinical benefit from the HER2–HER3 dimerization inhibitor.

5.2.5 Immunological Biomarkers of Cancer

Improvements in techniques have enabled demonstration of tumor-infiltrating lymphocytes (TILs) in different tumor compartments. TILs are associated with improved prognosis in cancer. As immunological biomarkers, TILs have an important role in determining outcomes of clinical interventions as intermediate endpoints. TILs are important as biomarkers for the development of cancer immunotherapies.

5.2.6 Metastatic Cancer Biomarkers

Biomarkers of metastases of various cancers have been investigated. Examples are given along with specific cancers. An example is as follows.

CUB domain-containing protein 1 (CDCP1) is a transmembrane protein that is highly expressed in stem cells and frequently overexpressed and tyrosine phosphorylated in cancer. CDCP1 promotes cancer cell metastasis. A study has shown that hypoxia induces CDCP1 expression and tyrosine phosphorylation in hypoxia-inducible factor (HIF)-2α—but not HIF-1α—dependent fashion (Emerling et al. 2013). shRNA knockdown of CDCP1 impairs cancer cell migration under hypoxic conditions, whereas overexpression of HIF-2α promotes the growth of tumor xenografts in association with enhanced CDCP1 expression and tyrosine phosphorylation. IHC analysis of tissue microarray samples from tumors of patients with clear cell renal cell carcinoma (RCC) shows that increased CDCP1 expression correlates with decreased overall survival. Together, these data support a critical role for CDCP1 as a unique HIF-2α target gene involved in the regulation of cancer metastasis, and suggest that CDCP1 is a biomarker and potential therapeutic target for metastatic cancers.

5.2.7 Tumor Microvesicles or Exosomes

Exosomes are small vesicles (50–100 nm) secreted by almost all tissues; they represent their tissue origin. Tumor cells release an abundance of microvesicles containing a selected set of proteins and RNAs. It has been shown that tumor microvesicles (exosomes) also carry DNA, which reflects the genetic status of the tumor, including amplification of the oncogene c-myc (Balaj et al. 2011). This study also found amplified c-myc in serum microvesicles from tumor-bearing mice. Further, the authors found remarkably high levels of retrotransposon RNA transcripts, especially for some human endogenous retroviruses, such as LINE-1 and Alu retrotransposon elements, in tumor microvesicles, and these transposable elements could be transferred to normal cells. These findings expand the nucleic acid content of tumor microvesicles to include elevated levels of specific coding and noncoding RNA and DNA, mutated and amplified oncogene sequences, and transposable elements. Thus, tumor microvesicles or exosomes contain a repertoire of genetic information available for horizontal gene transfer and potential use as blood biomarkers for cancer. The value of exosomes as biomarkers for prostate cancer and glioblastoma multiforme (GBM) is described in sections dealing with these tumors.

5.3 Technologies for Detection of Cancer Biomarkers

5.3.1 Proteomic Technologies for Detecting Biomarkers of Cancer

Proteins may be actively secreted or released by the tumor cells as a result of necrosis or apoptosis and released into the circulation. They change the protein profile. The difference in signal intensities may be detected by comparison with sera from normal individuals. A scheme for the role of proteomics in discovery of cancer biomarkers is shown in Fig. 5.1. Some of the technologies are described briefly in the following sections.

5.3.1.1 2D PAGE

2D PAGE followed by protein identification using MS has been the primary technique for biomarker discovery in conventional proteomic analyses. This technique is uniquely suited for direct comparisons of protein expression and has been used to identify proteins that are differentially expressed between normal and tumor tissues in various cancers, such as liver, bladder, lung, esophageal, prostate, and breast.

Advantages of 2D PAGE for discovery of biomarkers are as follows: (1) it is a tested and reliable method, (2) it can identify markers directly, and (3) it is reproducible and quantitative when combined with fluorescent dyes. Disadvantages of

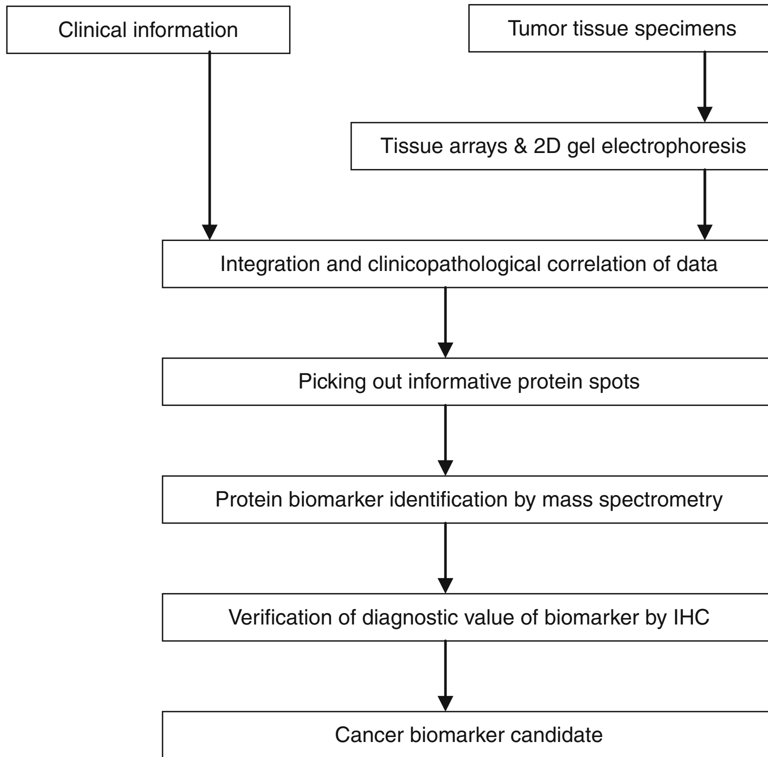

Fig. 5.1 Role of proteomics in the discovery of cancer biomarkers. © Jain PharmaBiotech

the use of 2D PAGE for this purpose are as follows: (a) it requires a large amount of protein as starting material making it unreliable for detecting and identifying low-abundance proteins, (b) early-stage cancers are often small and contamination from surrounding stromal tissue that is present in the specimen can confound the detection of tumor-specific markers, and (c) it has low sensitivity. The sensitivity can be improved by laser capture microdissection (LCM).

5.3.1.2 Antibody-Based Detection of Protein Biomarkers

T regulatory cells (Treg) expressing the FOXP3 transcription factor maintain immunological self-tolerance and enable tumor cells to escape immunosurveillance. FOXP3 is a biomarker in human malignancies and antibodies used to detect FOXP3 protein expression. FOXP3 can be used in routine clinical practice to provide both diagnostic and prognostic information, but the methods and reagents used to detect FOXP3 have a significant effect on the robustness of results (Bignone and Banham 2008).

The use of antibody microarrays continues to grow rapidly due to the recent advances in proteomics and automation and the opportunity this combination creates for high-throughput multiplexed analysis of protein biomarkers. However, a primary limitation of this technology is the lack of PCR-like amplification methods for proteins. Therefore, to realize the full potential of array-based protein biomarker screening, it is necessary to construct assays that can detect and quantify protein biomarkers with very high sensitivity, in the femtomolar range, and from limited sample quantities. Scientists at BioForce Nanosciences Inc. have described the construction of ultramicroarrays, combining the advantages of microarraying including multiplexing capabilities, higher throughput, and cost savings, with the ability to screen very small sample volumes (Nettikadan et al. 2006). Antibody ultramicroarrays for the detection of IL-6 and PSA, a widely used biomarker for prostate cancer screening, were constructed. These ultramicroarrays were found to have a high specificity and sensitivity with detection levels using purified proteins in the attomole range. Using these ultramicroarrays, they were able to detect PSA secreted from just four cells in 24 h. Cellular PSA could also be detected from the lysate of an average of just six cells. This strategy should enable proteomic analysis of materials that are available in very limited quantities such as those collected by LCM.

5.3.1.3 Aptamer-Based Molecular Probes for Cancer Biomarker Discovery

Aptamers (derived from the Latin word "aptus" = fitting) are short DNA or RNA oligomers, which can bind to a given ligand with high affinity and specificity due to their particular 3D structure and thereby antagonize the biological function of the ligand. Aptamers are used to detect the protein signatures of cells. This is based on the tendency of short DNA molecules—oligonucleotides—to fold into shapes that bind to specific proteins. The aptamer hugs its protein in the same way as an antibody embraces a specific antigen. The technology can be used with biochips. Aptamer technology may provide a method for monitoring protein changes in the blood that can echo the onset of carcinogenesis, for example, in women with genetic risk of breast cancer associated with BRCA1 dysfunction.

A strategy using cell-based aptamer selection has been developed to exploit the differences at the molecular level between any two types of cells for the identification of molecular signatures on the surface of targeted cells (Shangguan et al. 2006). A group of aptamers was generated for the specific recognition of leukemia cells. The selected aptamers can bind to target cells with an equilibrium dissociation constant (K_d) in the nanomolar-to-picomolar range. The cell-based selection process is simple, fast, straightforward, and reproducible, and, most importantly, can be done without prior knowledge of target molecules. The selected aptamers can specifically recognize target leukemia cells mixed with normal human bone marrow aspirates and can also identify cancer cells closely related to the target cell line in real clinical specimens. The cell-based aptamer selection holds a great promise in developing specific molecular probes for cancer diagnosis and cancer biomarker discovery.

5.3.1.4 Cancer Immunomics to Identify Autoantibody Signatures

Cancer immunomics has been used to identify autoantibody signatures produced in response to the presence of either breast or CRC. Serological proteome analysis (SERPA) is performed by 2D GE, immunoblotting, image analysis, and MS (Hardouin et al. 2007). Alternatively, to identify the antigens recognized by the autoantibodies of cancer patients, an approach has been developed that combines 2D immunoaffinity chromatography, enzymatic digestion of the isolated antigens, nanoflow separation of the resulting peptides, and identification: MAPPing (multiple affinity protein profiling). By these approaches both proteins recognized by autoantibodies independently of a cancer status are identified as well as a limited number of proteins reacting preferentially with cancer sera.

5.3.1.5 Desorption Electrospray Ionization for Detection of Cancer Biomarkers

A modified MS technique, desorption electrospray ionization (DESI), involves aiming a fine water mist at a surface with a pencil-sized tube that also sucks up the fluid after the droplets have mixed with the material in the sample (Wiseman et al. 2005). Whereas ordinary MS is both time- and labor-intensive, DESI not only is portable, but they can also determine the chemical composition of an unprepared sample within 5 s. Thus, DESI can detect chemical signature of cancer in liver tissue. The technique can tell the difference between diseased and non-diseased regions of tissue samples within a few seconds. Another advantage of DESI is that it can detect lipid biomarkers, whereas conventional MS is good at detecting protein biomarkers. Cancerous regions possess higher levels of certain lipid molecules, which could indicate a significant relationship between lipids and tumor proliferation. The devices might one day prove useful in helping surgeons ensure that all of the tumor is destroyed before a patient leaves surgery and also to identify other potential tumor sites in the tissue that are indistinguishable to the naked eye. It would help physicians determine how well a drug is working in different organs of the body. Analysis of different regions in a tissue sample would facilitate evaluation of the mechanism of its drug action and its effectiveness.

5.3.1.6 Detection of Circulating Nucleosomes in Serum of Cancer Patients

In the nucleus of eukaryotic cells, DNA is associated with several protein components and forms complexes known as nucleosomes. During cell death, particularly during apoptosis, endonucleases are activated that cleave the chromatin into multiple oligo- and mononucleosomes. Subsequently, these nucleosomes are packed into apoptotic bodies and are engulfed by macrophages or neighboring cells. In cases of high rates of cellular turnover and cell death, they also are released into the circulation

and can be detected in serum or plasma by Cell Death Detection ELISAplus (CDDE from Roche Diagnostics). As enhanced cell death occurs under various pathological conditions, elevated amounts of circulating nucleosomes are not specific for any benign or malignant disorder. However, the course of change in the nucleosomal levels in circulation of patients with malignant tumors during chemotherapy or radiotherapy is associated with the clinical outcome and can be useful for the therapeutic monitoring and the prediction of the therapeutic efficacy.

5.3.1.7 Detection of Tumor Markers with ProteinChip Technology

The ProteinChip Biomarker System (Vermillion) was developed for the Expression Difference Mapping™ of several hundreds of samples per day in a single uncomplicated platform with software support for the construction of multimarker predictive models. The Interaction Discovery Mapping™ platform was next introduced for the investigation of protein-binding partners of possible importance in diagnosis and therapy. SELDI-based ProteinChip technology has been used for the detection of tumor biomarkers (Wiesner 2004). The multimarker system has been shown to be superior over the single marker strategy and is faster. This system has been used for the detection of biomarkers of cancers of several organs including the prostate, ovary, breast, and lungs.

Surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) MS of platelet extracts for proteomic profiling shows increased amounts of angiogenic regulatory proteins such as VEGF and endostatin in platelet but not in plasma. This is a selective sequestration process and not a simple association with the platelet surface. This novel property of platelets detects human cancers of a microscopic size undetectable by any presently available diagnostic method. This is more inclusive than a single biomarker because it can detect a wide range of tumor types and tumor sizes. Relative changes in the platelet angiogenic profile permit the tracking of a tumor throughout its development, beginning from an early in situ cancer.

5.3.1.8 eTag Assay System for Cancer Biomarkers

The eTag assay system (Monogram Biosciences) is a high performance, high-throughput system for the study of tens to hundreds of genes, proteins, and cell-based antigens across thousands of samples. The system makes it possible for researchers to adopt a systems biology approach toward studies of gene expression and protein expression and for applications such as cell signaling and pathway activation, protein–protein interaction, and cell receptor binding. The system uses Monogram Biosciences' proprietary eTag reporters to multiplex the analysis of genes and/or proteins from the same sample. Specific molecular-binding events result in the release of electrophoretically distinct eTag reporters, which are then resolved to provide precise, sensitive quantitation of multiple analytes directly from cell lysates.

The eTag assay system is ideally suited to analysis of complex biology and medicine, such as that seen in oncology. These unique assays can precisely measure many types of pathway biomarkers simultaneously—using small samples, such as those obtained from standard tumor biopsies. Monogram Biosciences is collaborating with Norris Comprehensive Cancer Center at the University of Southern California, Los Angeles to identify and characterize novel clinical biomarkers for breast cancer. These biomarkers could be used to correlate disease type and progression, resulting in improved treatment. Novel eTag assays for unique protein biomarkers such as receptor complexes and phosphorylation events will be developed to focus on profiling epidermal growth factor receptor (EGFR) family signal transduction pathways. Further research will be aimed at applying eTag assays to retrospective analysis of patient samples for validation and diagnostic development. Knowledge gained from these studies will improve the prognosis of cancer patients by better understanding of treatments that will effect disease progression in specific patients. It is expected that these innovative eTag assays will enable the development of a broad range of biomarker-based personalized medicines to improve treatment for cancer.

5.3.1.9 Glycoprotein Biomarkers of Cancer

Changes in N-linked glycosylation are known to occur during the development of cancer. For example, increased branching of oligosaccharides has been associated with metastasis and has been correlated to tumor progression in human cancers of the breast, colon and melanomas. Increases in core fucosylation have also been associated with the development of hepatocellular carcinoma (HCC). Chronic infection with the hepatitis B virus is associated with more than 55 % of all cases of HCC. Increased levels of core fucosylation can be observed via glycan analysis of total serum and are associated with the development of HCC. In a blinded study, the serum glycoproteins derived from people diagnosed with HBV-induced liver cancer were found to possess a dramatically higher level of fucosylation (Comunale et al. 2006). This change occurs on both immunoglobulin molecules and other serum glycoproteins. Targeted glycoproteomic analysis was used to identify those glycoproteins that are hyperfucosylated in cancer. In total, 19 proteins were found to be hyperfucosylated in cancer. These proteins are potential biomarkers of cancer.

Gels prepared by biomolecular imprinting using lectin and antibody molecules as ligands have been used for recognition for tumor-specific marker glycoproteins (Miyata et al. 2006). The glycoprotein-imprinted gels prepared with minute amounts of cross-linkers can dynamically recognize tumor-specific marker glycoproteins by lectin and antibody ligands and induce volume changes according to the glycoprotein concentration. The glycoprotein-imprinted gels shrink in response to a target glycoprotein, but nonimprinted gels expand. The glycoprotein-responsive shrinking of the imprinted gel is caused by formation of lectin-glycoprotein-antibody complexes that act as reversible cross-linking points. Glycoprotein-imprinted gels only shrink when both lectin and antibody in the gels simultaneously recognize the saccharide and peptide chains of the target glycoprotein. As shrinking behavior of

biomolecularly imprinted gels in response to glycoproteins enables the accurate detection and recognition of tumor-specific marker glycoproteins, they have many potential applications as smart devices in sensing systems and for molecular diagnostics of cancer.

5.3.1.10 HER-2/Neu Oncoprotein as Biomarkers for Cancer

HER-2/neu oncoprotein has been widely studied for many years and has been shown to play a pivotal role in the development and progression of breast cancer. HER-2/neu has been shown to be an indicator of poor prognosis with patients exhibiting aggressive disease, decreased overall survival, and a higher probability of recurrence of disease. As evidenced by numerous published studies, elevated levels of HER-2/neu (also referred to as overexpression) are found in about 30 % of women with breast cancer. Determination of a patient's HER-2/neu status may be valuable in identifying whether that patient has a more aggressive disease and would, thus, derive substantial benefit from more intensive or alternative therapy regimens. Elevated levels of HER-2/neu are found not only in breast cancer but also in several other tumor types including prostate, lung, pancreatic, colon, and ovarian cancers.

Some studies suggest that in certain breast cancer patients, persistently rising HER-2/neu values may be associated with aggressive cancer and poor response to therapy, while decreasing HER-2/neu levels may be indicative of effective therapy. The clinical utility of the serum test as a prognostic indicator has not yet been fully established but is under investigation.

Traditional HER-2/neu testing is generally limited to tissue from primary breast cancer and does not provide information regarding the HER-2/neu status in women with recurrent, metastatic breast cancer (MBC). The introduction of microtiter plate ELISA HER-2/neu testing (Bayer Diagnostics) using a serum sample now offers a less invasive diagnostic tool and provides a current assessment of a woman's HER-2/neu status over the course of disease.

IHC analysis of HER2/neu in breast carcinoma is a useful predictor of response to therapy with trastuzumab when strongly positive. Negative immunostaining is highly concordant with a lack of gene amplification by fluorescent in situ hybridization (FISH). Most weakly positive overexpressors are false-positives on testing with FISH. Thus, screening of breast carcinomas with IHC and confirmation of weakly positive IHC results by FISH is an effective evolving strategy for testing HER2/neu as a predictor of response to targeted therapy.

5.3.1.11 Humoral Proteomics

The repertoires of serum autoantibodies differ between healthy persons and cancer patients. In healthy individuals these autoantibodies are directed against a limited number of self-proteins, but in cancer patients the antibody repertoires are much further expanded with a wider range of reactivities against other proteins. Although cancer patients clearly mount humoral immune responses, they are not very

effective in preventing the progression of the disease. The presence of these new and abnormal antibody specificities indicates their potential as novel tools for early detection of cancer before clinical manifestations. Proteomic technologies, with their unique ability to identify both tumor antigens and their cognate serum autoantibodies, hold great promise in facilitating the development of early detection kits and possibly also as conduits for the isolation of tumor antigens for immunotherapy (Shoshan and Admon 2007).

5.3.1.12 Laser Capture Microdissection

The introduction of LCM has greatly improved the specificity of 2D PAGE for biomarker discovery, as it provides a means of rapidly procuring pure cell populations from the surrounding heterogeneous tissue and also markedly enriches the proteomes of interest.

5.3.1.13 Membrane-Type Serine Protease-1

The cell surface protease membrane-type serine protease-1 (MT-SP1), also known as matriptase, is often upregulated in epithelial cancers. A study has shown that MT-SP1 is active on cancer cells and that its activity may be targeted in vivo for tumor detection (Darragh et al. 2010). A proteolytic activity assay with several MT-SP1-positive human cancer cell lines showed that MT-SP1 antibodies that inhibit recombinant enzyme activity in vitro also bind and inhibit the full-length enzyme expressed on cells. In contrast, in the same assay, MT-SP1-negative cancer cell lines were inactive. Fluorescence microscopy confirmed the cell surface localization of labeled antibodies bound to MT-SP1-positive cells. To evaluate in vivo targeting capability, fluorescently labeled antibodies were administered to mice bearing tumors that were positive or negative for MT-SP1. Antibodies localized to MT-SP1-positive tumors enabling visualization of MT-SP1 activity, whereas MT-SP1-negative tumors were not visualized. These findings define MT-SP1 activity as a useful biomarker for epithelial cancers using a noninvasive antibody-based method.

5.3.1.14 Phage Display Technology

Phage display technology utilizes combinatorial libraries of proteins expressed on phage particles that can be selected for specific binding to cancer cells (Samoylova et al. 2006). Such cancer-specific molecules can be used in a variety of applications, including identification of cell-specific targeting molecules, identification of cell surface biomarkers, profiling of specimens obtained from individual cancer patients, and the design of peptide-based anticancer therapeutics for personalized treatments. Peptide phage display strategies can target cell surfaces because many biomarkers important in cancer are differentially expressed molecules located on the outside of the cell membranes.

A novel method called ADEPPT (accentuation of differentially expressed proteins using phage technology) can identify proteins that are produced in different amounts in diseased tissue compared with healthy tissue (Suber et al. 2004). The study used a large "library" with thousands of strains of the bacteriophage known as M13. Each strain of M13 makes a different peptide, which binds to a specific protein. By using a large bacteriophage library, the researchers had the best possible chance of detecting all the proteins that were produced at varying levels in different tissue samples. This technique can rapidly determine differences between lung cancer tissue and normal lung tissue by measuring subtle variations in the proteins they produce. ADEPPT can pick up proteins in lung cancer that are overlooked by more conventional methods of protein profiling. This method may ultimately enable researchers to detect proteins responsible for all types of cancer and potentially assist them in finding better drug targets to treat various diseases. This information could also be used for early detection of cancers, e.g., by testing for elevated levels of proteins in the blood, which could potentially improve the chance of successful treatment. Further development of the ADEPPT method is needed, but initial indications are that it may complement existing methods such as 2D gel electrophoresis by detecting less abundant proteins.

5.3.1.15 Proteomic Analysis of Cancer Cell Mitochondria

A combination of reverse-phase protein microarray with radiolabeled glucose metabolic studies has shown that there is a specific association between altered cytochrome c oxidase subunit levels and altered metabolism in cancer cells (Krieg et al. 2004). Mutations in mitochondrial DNA have been frequently reported in cancer cells. Significance of gene expression patterns is not established yet. Roles of proteomics in the study of mitochondrial proteome in cancer are as follows:

- Identification of abnormally expressed mitochondrial proteins in cancer cells is possible by mitochondrial functional proteomics.
- Proteomics can identify new markers for early detection and risk assessment, as well as targets for therapeutic intervention.

5.3.1.16 Proteomic Technologies for Detection of Autoimmune Biomarkers

There is considerable evidence for an immune response to cancer in humans, as demonstrated in part by the identification of autoantibodies against a number of tumor-associated antigens in sera from patients with different cancer types (Desmetz et al. 2008). Thus, identification of tumor-associated antigens and their associated antibodies is a promising strategy to find relevant biomarkers. Proteomic approaches such as SEREX and SERPA have enabled identification of a large

numbers of antigens and their cognate autoantibodies. They have many advantages for identification of relevant autoantigens in different types of cancer. However, they are also time-consuming and lack sensibility and specificity. To circumvent these drawbacks, new proteomic techniques, based on protein or antibody arrays, allow high-throughput analysis of multiple targets in a single experiment. Specific combinations of biomarkers should be identified, as they are more effective for detection of cancer compared to a single biomarker. These approaches are promising for discovery of cancer biomarkers, but also need to be further validated for clinical application on large populations.

In cancer cells, cyclic AMP-dependent protein kinase (PKA) is secreted into the conditioned medium. This PKA, designated as extracellular protein kinase A (ECPKA), is markedly upregulated in the sera of patients with cancer. The currently available tumor biomarkers are based on the antigen determination method and lack specificity and sensitivity. An ECPKA autoantibody detection method has been described for a universal biomarker that detects cancer of various cell types (Nesterova et al. 2006). In serum specimens, the presence of autoantibody directed against ECPKA was highly correlated with cancer. High anti-ECPKA autoantibody titers were found in the sera of patients with various cancers, whereas low or negative titers were found in the control group. The autoantibody enzyme immunoassay exhibited 90 % sensitivity and 88 % specificity, whereas the enzymatic assay exhibited 83 % sensitivity and 80 % specificity. These results show that the autoantibody method distinguished between patients with cancer and controls better than the antigen method could. Thus, autoantibody ECPKA is a universal serum biomarker for cancers of various cell types.

5.3.1.17 SELDI-TOF MS

SELDI-TOF MS is an important tool for the rapid identification of cancer-specific biomarkers and proteomic patterns in the proteomes of both tissues and body fluids. It is useful in high-throughput proteomic fingerprinting of cell lysates and body fluids that uses on-chip protein fractionation coupled to time-of-flight separation. Within minutes, sub-proteomes of a complex milieu such as serum can be visualized as a proteomic fingerprint or "barcode." SELDI technology has significant advantages over other proteomic technologies in that the amounts of input material required for analysis are miniscule compared with more traditional 2D PAGE approaches. A number of studies have used SELDI technology to identify single disease-related biomarkers for several types of cancer. For example, a modified, quantitative SELDI approach has been used to show that the levels of serum prostate-specific membrane antigen (PSMA) are significantly higher in patients with prostate cancer than in those with benign disease.

5.3.1.18 Serum Proteome Analysis for Early Detection of Cancer

Proteome analysis has been used for the identification of biomarkers or biomarker patterns that may enable early diagnosis of cancer. This tool is of special interest, since it allows for the identification of tumor-derived secretory products in serum or other body fluids. In addition, it may be used to detect reduced levels or loss of proteins in the serum of cancer patients that are present in noncancer individuals. These changes in the serum proteome may result from cancer-specific metabolic or immunological alterations, which are, at least partly, independent of tumor size or mass, thereby facilitating early discovery.

5.3.1.19 Triple Quadrupole MS for Detection of Mutant Proteins

Gene products resulting from somatic mutations are meaningful protein biomarkers, because they are not merely associated with tumors but are responsible for carcinogenesis. Altered protein products resulting from somatic mutations can be identified directly and quantified by MS. Peptides expressed from normal and mutant alleles have been detected by selected reaction monitoring (SRM) of their product ions using a triple quadrupole MS (Wang et al. 2011). As an example of this approach, the authors demonstrated that it is possible to quantify the number and fraction of mutant Ras protein present in cancer cell lines. There were an average of 1.3 million molecules of Ras protein per cell, and the ratio of mutant to normal Ras proteins ranged from 0.49 to 5.6. Similarly, they found that mutant Ras proteins could be detected and quantified in clinical specimens such as colorectal and pancreatic tumor tissues as well as in premalignant pancreatic cyst fluids. In addition to answering basic questions about the relative levels of genetically abnormal proteins in tumors, this approach could prove useful for diagnostic applications.

5.3.1.20 Targeted MS for Validation of Cancer Biomarkers in Plasma

A data-dependent triage process was used to prioritize a subset of putative plasma biomarkers from >1,000 candidates previously identified using a mouse model of breast cancer (Whiteaker et al. 2011). The team used a comparable targeted MS that relied on "accurate inclusion mass screening" (AIMS) and another targeted method known as SRM-MS, to sift through candidate biomarkers. Thirty-six proteins were verified as being elevated in the plasma of tumor-bearing animals. The final candidate biomarkers were highly credentialed so they had a high probability of being real biomarkers. The analytical performance of this pipeline suggests that it should support the use of an analogous approach with human samples. Although the biological variation among humans will undoubtedly be greater than that among mice, the preanalytic and analytical variations associated with the technologies are the same regardless of which species is used.

5.3.1.21 Tissue Proteomics for Discovery of Cancer Biomarkers

The molecular complexity of tissue and the in vivo inaccessibility of cells within solid tumors hinder efforts to discover new diagnostic biomarkers useful for noninvasive tumor-specific molecular imaging. Subcellular fractionation of tissue, subtractive proteomic analysis, bioinformatics, and expression profiling have enabled identification of several biomarkers induced in solid tumors at the tissue–blood interface that are accessible to agents injected intravenously. Immunotargeting and penetration of single organs and solid tumors can be validated by molecular imaging. This approach can identify biomarkers expressed on vascular endothelium and its specialized transport vesicles (caveolae) in multiple human and rodent tumors including primary and metastatic lesions found in the liver, brain, breast, kidney, lung, intestine, and prostate. Mapping tissue- and disease-modulated endothelial cell surface and caveolar proteins reveals promising biomarkers for imaging and therapy of solid tumors.

5.3.2 Metabolomic Biomarkers of Cancer

Compared to the cancer genome and the cancer proteome, there are few studies on the cancer metabolome. The importance of metabolomics becomes obvious if one looks at the pathways involved in cancer. Tumor cells respond to growth signals by the activation of protein kinases, altered gene expression, and changes in cellular function. The transformed cells show unique anabolic characteristics, which include increased and preferential utilization of glucose through the nonoxidative steps of the pentose cycle for nucleic acid synthesis but limited de novo fatty acid synthesis and tricarboxylic acid (TCA) cycle glucose oxidation. This primarily nonoxidative anabolic profile reflects an undifferentiated highly proliferative cell phenotype and serves as a reliable metabolic biomarker to determine cell proliferation rate and the level of cell transformation/differentiation in response to drug treatment. Novel drugs effective in particular cancers exert their anticancer effects by inducing significant reversions of a few specific non-oxidative anabolic pathways. Cell transformation of various mechanisms is sustained by a unique disproportional substrate distribution between the two branches of the pentose cycle for nucleic acid synthesis, glycolysis and the TCA cycle for fatty acid synthesis and glucose oxidation. This can be demonstrated by the broad labeling and unique specificity of [1,2-(13) C(2)]glucose to trace a large number of metabolites in the metabolome as biomarkers. Stable isotope-based dynamic metabolic profiles serve the drug discovery process by providing a powerful new tool that integrates the metabolome into a functional genomic approach to developing new drugs. It can be used in screening kinases and their metabolic targets, which can therefore be more efficiently characterized, speeding up and improving drug testing, approval, and labeling processes by saving trial-and-error-type study costs in drug testing.

5.3.2.1 Magnetic Resonance for Detecting Metabolomics Biomarkers of Cancer

When using MR metabolomics as a clinical tool in oncology, it is essential to differentiate between metabolic profiles of cancer and normal tissue. High-resolution magic angle spinning (HR-MAS), in combination with multivariate analysis, clearly discriminates the metabolic profiles of cancer tissue and normal and/or adjacent tissue in different cancer types such as breast, colorectal, prostate, and cervical (Bathen et al. 2010). The most prominent differences are found in metabolites such as the choline-containing compounds (ChoCC), glycine and glucose. Multivariate analysis combines information from all metabolites detected in the tissue samples simultaneously. By interpreting these results, it is possible to identify single metabolites or ratios of metabolites that are the most differently expressed. In this way, possible biomarkers can be detected and information about specific pathways obtained.

5.3.2.2 Choline Phospholipid Biomarkers of Cancer

Choline phospholipid metabolism is altered in several cancers. HR-MAS is useful but magnetic resonance imaging (MRI) and positron emission tomography (PET) can also be used for choline metabolism-based diagnosis of cancer. The choline metabolite profile of tumors is characterized by elevation of phosphocholine and phosphocholine-containing compounds (Glunde and Serkova 2006). They can be used as endogenous biomarkers of cancer or for monitoring the response of cancer to treatment. The enzymes directly causing this elevation such as choline kinase and phospholipases C as well as D may provide targets for anticancer drugs. Signal transduction pathways that are activated in cancer such as those mediated by receptor tyrosine kinases and EGFR also provide targets for drugs. MRS has been used to study pharmacodynamic markers of the choline kinase inhibitor MN58b in human carcinoma models (Al-Saffar et al. 2006). Inhibition of choline kinase by MN58b resulted in altered phospholipid metabolism both in cultured tumor cells and in vivo. Phosphocholine levels were found to correlate with choline kinase activities. The decrease in phosphocholine, total choline, and phosphomonoesters are potential noninvasive pharmacodynamic biomarkers for determining tumor response following treatment with choline kinase inhibitors.

5.3.2.3 Hypoxia-Inducible Factor-1

Tumor hypoxia is well recognized in oncology to be a key factor resulting in treatment resistance and poor prognosis. Hypoxia leads to the expression of a number of gene products that are involved in tumor progression, invasion, and metastasis formation. The most important of these proteins is thought to be hypoxia-inducible factor-1 (HIF-1), which appears to be a master regulator of the cellular response to

hypoxia. HIF-1 pathway, which enables cells to sense and respond to hypoxia, is overexpressed in many cancers. HIF-1 is a transcription factor that upregulates numerous genes involved in processes such as glucose metabolism, glycolysis, pH regulation, cellular proliferation, matrix metabolism, and regulation of blood vessels. HIF-1 is being explored as an attractive target for drug discovery.

HIF-1 expression is associated with a poor prognosis and treatment response in a number of tumor sites. There is some evidence that the HIF-1 pathway might be involved in gastric carcinogenesis. Immunohistochemical expression of HIF-1 target genes is associated with a poor prognosis of gastric cancer. Targeted inhibition of HIF-1 has been shown to inhibit the growth of gastric tumors in animals (Griffiths et al. 2005). Increased understanding of the importance of hypoxia and the HIF-1 pathways may, therefore, hold the key to prevention strategies, improved selection of patients for adjuvant therapy, and new treatments for the disease.

Expression of endogenous markers of hypoxia for the HIF-1/HIF-2 pathway is strongly associated with radiotherapy failure. Using immunohistochemical methods, it is possible to identify subgroups of head and neck squamous cell carcinoma (HNSCC) patients who are highly curable with radiotherapy or who are excellent candidates for clinical trials on hypoxia-targeting drugs in two distinct pathways (Koukourakis et al. 2006).

5.3.2.4 Detection of Drug Resistance in Cancer by Metabolic Profiling

Acquired resistance to imatinib mesylate is an increasing and continued challenge in the treatment of BCR–ABL tyrosine kinase-positive leukemias as well as gastrointestinal stromal tumors. Stable isotope-based dynamic metabolic profiling (SIDMAP) studies conducted in parallel with the development and clinical testing of imatinib revealed that this targeted drug is most effective in controlling glucose transport, direct glucose oxidation for RNA ribose synthesis in the pentose cycle, as well as de novo long-chain fatty acid synthesis (Serkova and Boros 2005). Thus, imatinib deprives transformed cells of the key substrate of macromolecule synthesis, malignant cell proliferation, and growth. Tracer-based MRS studies revealed a restitution of mitochondrial glucose metabolism and an increased energy state by reversing the Warburg effect, consistent with a subsequent decrease in anaerobic glycolysis. Recent in vitro SIDMAP studies that involved myeloid cells isolated from patients who developed resistance against imatinib indicated that non-oxidative ribose synthesis from glucose and decreased mitochondrial glucose oxidation are reliable metabolic signatures of drug resistance and disease progression. There is also evidence that imatinib-resistant cells utilize alternate substrates for macromolecule synthesis to overcome limited glucose transport controlled by imatinib. The main clinical implications involve early detection of imatinib resistance and the identification of new metabolic enzyme targets with the potential of overcoming drug resistance downstream of the various genetic and BCR–ABL expression-derived mechanisms. Metabolic profiling is an essential tool used to predict, clinically detect, and treat targeted drug resistance. This need arises from the fact that

targeted drugs are narrowly conceived against genes and proteins but the metabolic network is inherently complex and flexible to activate alternative macromolecule synthesis pathways that targeted drugs fail to control.

5.3.2.5 Plasma-Free Amino Acids Profiling in Cancer

Profiling of plasma-free amino acids (PFAAs) is a promising approach for early detection of cancer. Focused metabolomic approach with PFAAs overcomes some of the problems associated with metabolomic profiling of cancer that include the need for measuring a huge number of metabolites, data-redundancy problems, and high cost. In a study using large number of plasma samples from cancer patients, PFAA levels were measured using HPLC and ESI-MS (Miyagi et al. 2011). Univariate analysis revealed significant differences in the PFAA profiles between the controls and the patients with any of the five types of cancer—lung, gastric, colorectal, breast, or prostate—even in patients with asymptomatic early-stage disease. These findings indicate that PFAA profiling from a single blood sample has considerable potential for improving cancer screening and diagnosis and understanding disease pathogenesis.

5.3.2.6 Urinary Metabolomic Biomarkers of Cancer

Metabolomic techniques have been used to identify metabolites in urine of patients with kidney cancer, but their levels differ from the same metabolites in nonkidney cancer patients (Kim et al. 2011). These authors found that quinolinate, 4-hydroxybenzoate, and gentisate are differentially expressed at a false discovery rate of 0.26, and these metabolites are involved in common pathways of specific amino acid and energy metabolism, consistent with high tumor protein breakdown and utilization. When added to four different (three kidney cancer-derived and one "normal") cell lines, several of the significantly altered metabolites, quinolinate, α-ketoglutarate, and gentisate, showed increased or unchanged cell proliferation that was cell line dependent. Further evaluation as well as validation of the specific potential biomarkers using a larger sample size will be required before use of these biomarkers for diagnosis and therapy of kidney cancer.

Identification of volatile organic metabolites (VOMs) as biomarkers that can accurately diagnose the onset of cancer using noninvasively collected urine specimens is ideal for early detection. This approach has been applied to diagnosis of various cancers. A study of the urinary metabolomic profile of breast cancer patients and healthy individuals as controls has explored VOMs as potential biomarkers in breast cancer diagnosis at early stage (Silva et al. 2012). Solid-phase microextraction (SPME) using CAR/PDMS sorbent combined with GC–MS was applied to obtain metabolomic patterns. Ketones and sulfur compounds were the chemical classes with highest contribution for both groups. Results showed that excretion values of six VOMs among the total of 79 detected were found to be statistically different. A significant increase in the peak area of (−)-4-carene, 3-heptanone,

1,2,4-trimethylbenzene, 2-methoxythiophene, and phenol, in VOMs of cancer patients relatively to controls was observed. Statistically significant lower abundances of dimethyl disulfide were found in cancer patients. Another study found that urinary biomarkers disturbed in several metabolic pathways of epithelial ovarian cancer patients included those associated with nucleotide metabolism, histidine metabolism, tryptophan metabolism, and mucin metabolism (Zhang et al. 2013).

5.3.3 Epitomics for the Early Detection of Cancer

Efforts toward the development of early detection assays for cancer have traditionally depended on single biomarker molecules. Current technologies have been disappointing and have not resulted in diagnostic tests suitable for clinical practice. Using a high-throughput cloning method, a panel of epitopes/antigens that react with autoantibodies to tumor proteins in the serum of patients with ovarian cancer have been isolated (Draghici et al. 2005). Discovery of biomarker panels was directed in an unbiased fashion by cloning a large panel of epitopes or tumor antigens, rather than individual biomarkers without a previous notion of their function. The binding properties of these serum antitumor antibodies on microarrays and advanced bioinformatic tools led to a panel of diagnostic antigens. The sequences that were identified using this new technology will lead to the discovery of novel disease-related proteins that have diagnostic value for the presymptomatic detection of cancer. It has been demonstrated that this approach can detect these autoantibodies in the sera of stage I ovarian cancer patients. There are numerous advantages of employing serum antibodies as the analytes, not the least of which is the ability to rapidly adapt these assays to standard clinical platforms. This technology of global epitope/antigen profiling is referred to as "epitomics."

5.3.4 Detection of Biomarkers of DNA Methylation

Methylated DNA (meDNA) is a very stable carrier of epigenetic information that is directly involved in tumor formation and progression. Genes that are often methylated in tumors are termed tumor biomarkers because their methylation can be used to detect the disease. Utilization of methylated DNA markers is superior to reliance on other types of markers for numerous reasons, including the following:

- Methylation is directly responsible for regulation of many cancer genes.
- DNA is chemically and biologically more stable than RNA and many other types of biomarkers.
- The levels of gene methylation in cancer cells are very constant and not subject to fluctuations as seen for expressed products such as RNA, proteins, and metabolites.
- meDNA assays are inherently very sensitive (MethylPlex can detect as little as one cancer cell in a mixture with 10,000 normal cells).

- meDNA is a universal analyte for diagnostics, because the same technology, instrumentation, and data analysis methods can be used to detect many types of cancer and other diseases.

Some problems with DNA methylation analysis are as follows:

- Probability of methylation for each gene is less than 100 %.
- Clinical samples are heterogeneous.
- Quantitative assay of methylation is difficult.

Because of variations of methylation of genes in different types of cancer and various stages of the disease, the methylation of any single gene may not provide sufficient information. However, a test based on the pattern of methylation in a number of genes would have the high sensitivity and specificity required for an accurate diagnosis and prognosis. Use of a systematic biological screen has enabled identification of multiple genes that are methylated with high penetrance in primary lung, breast, colon, and prostate cancers (Shames et al. 2006). The cross-tumor methylation pattern observed for these novel biomarkers suggests that a partial promoter hypermethylation signature for these common malignancies has been identified. These data suggest that while tumors in different tissues vary substantially with respect to gene expression, there may be common features in their promoter methylation profiles that could form the basis for a new early detection screen for certain cancers.

Methyl-BEAMing technology enables absolute quantification of the number of methylated molecules in a sample (Li et al. 2009). Individual DNA fragments are amplified and analyzed either by flow cytometry or by next-generation sequencing, and as few as one methylated molecule can be enumerated in approximately 5,000 unmethylated molecules in DNA from plasma or fecal samples. Using methylated vimentin as a biomarker in plasma samples, methyl-BEAMing detected 59 % of cancer cases. In addition to diagnostic and prognostic applications, this digital quantification of rare methylation events should be applicable to preclinical assessment of new epigenetic biomarkers and quantitative analyses in epigenetic research. In early-stage CRC, sensitivity of detection by methyl-BEAMing technology is four times more than that obtained by assaying carcinoembryonic antigen (CEA). With stool samples, methyl-BEAMing detected 41 % of cancers and 45 % of advanced adenomas.

Changes in gene expression due to epigenetic regulation can be reversed by chemicals, and this approach opens up a novel approach in cancer treatment in addition to diagnosis. Methylation in noncancerous tissues is now attracting attention as a biomarker of risk of cancer and is emerging as a target for cancer prevention. Many of the tests are in development commercially and will be described briefly.

5.3.4.1 PCR with Bisulfite for Detecting DNA Methylation Biomarkers in Cancer

Several traditional and new PCR-based methods have been developed for detecting DNA methylation at single loci. All have characteristic advantages and disadvantages, particularly with regard to use in clinical settings. In order to detect

methylation patterns on DNA, one needs greater amounts of it than can be extracted from a small patient sample. In order to achieve this, one can increase the amount of DNA extracted from minute samples rather than use larger samples. Use of PCR for amplifying DNA results in the loss all information on the positions of methylcytosine.

To overcome the limitations of conventional PCR, Epigenomics Inc. uses a procedure that is based on modification of all non-methylated cytosines to a different base, uracil, by the chemical bisulfite. Uracil's hybridization behavior is identical to that of thymine. Thus, in DNA treated with bisulfite, methylcytosine can easily be detected by hybridizing to guanine. This enables the use of variations of established methods of molecular biology, most notably PCR, hybridization, oligonucleotide arrays, and mass spectrometry. The remaining cytosines are present in the sequence context 5′-cytosine-guanine-3′ (CpG). The total number of CpG positions in the human genome is ~40 million, up to 10 % of which are located in non-repetitive, relevant sections of DNA.

MDxHealth Inc.'s methylation-specific PCR (MSP) can rapidly assess the methylation status of virtually any group of CpG sites within a CpG island, independent of the use of methylation-sensitive restriction enzymes. An MSP assay entails initial modification of DNA by sodium bisulfite, converting all unmethylated, but not methylated, cytosines to uracil, and subsequent amplification with primers specific for methylated versus unmethylated DNA. MSP requires only small quantities of DNA, is sensitive to 0.1 % methylated alleles of a given CpG island locus, and can be performed on DNA extracted from paraffin-embedded samples. MSP eliminates the false-positive results inherent to previous PCR-based approaches, which relies on differential restriction enzyme cleavage to distinguish methylated from unmethylated DNA. Patent-protected MSP platform is the only scalable technology that enables a sensitive and specific detection of methylated genes in a background of normal cells, critical for early diagnosis or detection of micrometastases in serum, saliva, or sputum samples. The process employs an initial bisulfite reaction to modify the DNA, followed by PCR amplification with specific primers designed to distinguish methylated from unmethylated DNA. This specific alteration enables the detection of a few cancer cells embedded in otherwise normal tissue. This process is universal and can be applied to the detection of promoter hypermethylation of relevant tumor suppressor genes or any other cellular genes related to cancer. A practical aspect of this diagnostic marker strategy is the concentration on specific genes known to play an important role in tumorigenesis. This approach is far less labor intensive and more amenable to high-throughput screening than microarray assays.

Orion's MethylScreen® technology leverages biomarkers discovered using its MethylScope technology to develop a new class of oncology diagnostic kits. MethylScreen is a reliable enzyme-based real-time PCR technology that is compatible with testing platforms widely used in clinical laboratories. The sensitivity of MethylScreen assays enables Orion's scientists to measure unique qualities of epigenetic DNA that are indicative of disease progression. MethylScreen assays provide critical clinical information about disease progression using blood serum and other easily collected patient samples. MethylScreen is unique in that it is the only

platform that does not rely on bisulfite conversion, a harsh chemical process that has been shown to destroy more than 94 % of the tumor DNA in patient samples.

MethySYBR is a SYBR green-based PCR assay for the dual analysis of DNA methylation and CpG methylation density (Lo et al. 2009). MethySYBR begins with multiplex PCR to enable the simultaneous amplification of many discrete target alleles in a single reaction using bisulfite-converted DNA. In the second round of PCR, the specific methylated target is quantified from multiplex products using both nested methylation-independent and methylation-specific primer sets. Moreover, the use of SYBR green dye during quantitative PCR enables melting curve analysis of target amplicons to determine the methylation density of CpG sites on target alleles. To establish proof of principle, two cancer-specific methylated genes, RASSF1A and OGDHL, were assessed by MethySYBR. MethySYBR sensitively detected methylated alleles in the presence of a 100,000-fold excess of unmethylated allele. Furthermore, MethySYBR was shown to be capable of analyzing minute amounts of DNA from paraffin-embedded tissue. Therefore, the MethySYBR assay is a simple, highly specific, highly sensitive, high-throughput, and cost-effective method that is widely applicable to basic and clinical studies of DNA methylation.

PCR-based methods that use sodium bisulfite-treated DNA as a template are generally accepted as the most analytically sensitive and specific techniques for analyzing DNA methylation at single loci (Kristensen and Hansen 2009). A number of new methods, such as methylation-specific fluorescent amplicon generation (MS-FLAG), methylation-sensitive high-resolution melting (MS-HRM), and sensitive melting analysis after real-time methylation-specific PCR (SMART-MSP), now complement the traditional PCR-based methods and promise to be valuable diagnostic tools. In particular, the HRM technique shows great potential as a diagnostic tool because of its closed-tube format and cost-effectiveness.

5.3.4.2 MDScan™ Microarray Technology

GenomicTree's MDScan™ technology for the systematic and comprehensive genome-wide discovery of epigenetically silenced genes uses affinity-based methyl DNA enrichment, a bisulfite-free method, for selective enrichment of methylated DNA. The selectively isolated methyl DNA can be used for microarray analysis. This technology does not depend on bisulfite modification, restriction endonucleases, or specific antibodies and will lead to the discovery of novel methylation biomarkers for early detection of cancer, determination of tumor stage, risk of recurrence, and prediction of response to drug therapy.

5.3.4.3 Rubicon MethylPlex Technology

Rubicon Genomics believes that detection of methylated DNA in serum and urine is the best approach for developing practical noninvasive tests for cancer.

The challenge is to implement a test for those genes using a noninvasive protocol. Currently, there are two significant barriers to noninvasive testing:

1. meDNA tests require a large amount of cancer DNA, which is often not present in the blood or urine of cancer patients, especially during the initial stages of disease or upon recurrence.
2. meDNA tests use a special chemistry, called bisulfite conversion, and proprietary amplification technologies that make testing complicated and expensive.

The Rubicon MethylPlex technology has solved both of these problems by completely avoiding bisulfite chemistry and improving technical sensitivity by a factor of 10–100 times, so that the methylation of many genes can be reliably detected from serum and urine.

5.3.4.4 Epigenomics Marker Machine for DNA Methylation Biomarkers

Epigenomic AG has developed a proprietary technology consisting of a combination of chemically treated DNA, highly multiplexed amplifications, high-density arrays, and MALDI MS. This technology makes the detection of hundreds of thousands of DNA methylation signals a reality. These signals can be digitized into a long string of ones and zeros, creating a digital phenotype that reflects genetic activity in a particular cell or tissue, i.e., whether it is functioning normally or whether it is sick.

A large-scale genome-wide screening effort of all major human tumors for DNA methylation biomarkers in tissue and serum has led to the discovery of 200 such biomarkers. Epigenomics believes that methylation-based DNA biomarkers will allow the detection of disease much earlier than currently available diagnostics. This will allow physicians to develop the best treatment for existing disease, monitor the effects of treatment, and identify people at risk of developing disease. These results truly reveal the power of Epigenomics Marker Machine. Such novel DNA methylation biomarkers could form the backbone of future molecular diagnostics. Another fascinating finding is that dozens of these biomarkers are derived from genes not yet implicated in cancer development, making them highly interesting candidates as pharmaceutical targets. It is generally accepted that DNA methylation is involved in global transcriptional regulation of the human genome. Over-methylation can lead to decreased gene activity, for instance, blocking genes that protect against cancer. On the flip side, under-methylation of normally inactive genes often leads to their activation and acceleration of the disease process. Epigenomics expects to develop validated panels of informative DNA methylation positions for various types of cancer diagnostics such as differential diagnosis, treatment planning tests, monitoring tests, and serum-based early screening tests. The convenience, performance, and cost-effectiveness of these tests will potentially make them ideal for mass screening programs for all major cancer types.

5.3.4.5 SEQUENOM's Integrated Genetic Analysis Platform

Researchers at SEQUENOM have described an integrated genetic analysis platform based on MALDI-TOF MS to correlate SNPs with differences in allele-specific expression, and on the basis of this information to examine possible variations in DNA methylation patterns being causative of these differences. The group validated their approach using an established model based on allele-specific transcript levels of the human tumor protein 73, indicating the suitability of the SEQUENOM's MassARRAY platform for assaying both stable and dynamic variations at the DNA and RNA level.

5.3.4.6 Histone Deacetylase

Levels of histone acetylation are tightly modulated in normal cells, and alterations of their regulating mechanisms have been shown to be involved in carcinogenesis. A flow cytometric technique for detection of histone acetylation has been developed based on a specific MAb that recognizes acetylated histone tails (Ronzoni et al. 2005). Flow cytometric detection of acetylation status can successfully detect modifications induced by the histone deacetylase (HDAC) inhibitor treatment in vivo. Changes in acetylation levels during the cell cycle demonstrated a reproducible increase in histone acetylation during the replication phase that was subsequently decreased at the G2M entrance, thus paralleling the behavior of DNA replication and transcriptional activity. Multiparameter analysis of histone acetylation and expression of molecular markers, DNA ploidy, and/or cell cycle kinetics can provide a quick and statistically reliable tool for the diagnosis and evaluation of treatment efficacy in clinical trials using HDAC inhibitors. HDAC inhibitors induce differentiation of breast cancer cells and inhibit tumor growth. HDAC-1 protein expression was analyzed immunohistochemically on a tissue microarray containing biopsies from breast cancer patients (Krusche et al. 2005). HDAC-1 expression was correlated to steroid hormone receptor, Her2/neu, and proliferation status of tumors as well as to overall and disease-free survival. Multivariate analysis demonstrated that HDAC-1 is an independent prognostic marker and evaluation of HDAC-1 protein expression enables a more precise assessment of the prognosis of breast cancer patients. HDAC-1 expression analysis might be clinically useful to facilitate an individual, risk-directed, adjuvant systemic therapy in breast cancer patients.

5.3.4.7 Mucins as Epigenetic Biomarkers in Epithelial Cancers

Genes encoding mucins have been shown to be regulated by DNA methylation and histone modifications in epithelial cancer cells. These genes encode either secreted glycoproteins necessary for epithelial homeostasis or membrane-bound glycoproteins that participate in tumor progression. The important biological functions played by these large molecules in pathophysiology of the epithelia make them key

genes to target for new therapeutic strategies and new diagnostic and/or prognostic tools in cancer. Mucin genes may be regulated by miRNAs and also regulate miRNA activity. Epigenetic regulation of mucin genes has a great potential to provide new diagnostic as well as prognostic biomarkers, help tumor classification and form the basis of new therapeutic targets for the clinician and the pathologist (Van Seuningen and Vincent 2009).

5.3.5 Nanobiotechnology for Early Detection of Cancer to Improve Treatment

Cancer is easier to treat and less likely to develop drug resistance when treatment is started very early. Cancer cells in very early stages are less likely to have mutations that make them resistant to treatment. However, cancer cells themselves may be difficult to detect at an early stage, but they leave a fingerprint, i.e., a pattern of change in biomarker proteins that circulate in the blood. There may be 20–25 biomarkers, which may require as many as 500 measurements, all of which should be made from a drop of blood obtained by pinprick. Thus, nanoscale diagnostics will play an important role in this effort. Nanowire sensors in development can electronically detect a few proteins molecules along with other biomarkers that are early signs of cancer. Each nanowire in a set is coated with a different compound that binds to a particular biomarker and changes the conductivity of that can be measured. Thousands of such nanowires are combined on a single chip that enables identification of the type of cancer. Currently such a chip can detect between 20 and 30 biomarkers and can be used for the early diagnosis of brain cancer.

5.3.5.1 NP-Peptide Complexes for Detection of Cancer Biomarkers in Urine

Exogenously administered “synthetic biomarkers” composed of mass-encoded peptides conjugated to nanoparticles (NPs) have been developed for noninvasive urinary monitoring to detect cancer biomarkers (Kwong et al. 2012). The NP complexes accumulate at the tumor site, where matrix metalloproteinases (MMPs) from cancer cells are expected to cleave the NP-bound peptides, releasing them into the bloodstream. The peptides would then accumulate in the kidneys and be excreted in the urine, which could be analyzed using MS. The NPs are engineered to express ten different peptides, each with a specific corresponding MMP and size making them distinguishable by MS. In mouse models of liver cancer, these agents were shown to substantially improve early detection of cancer compared with current clinically used blood biomarkers. This method has potential for development as point-of-care diagnostics to detect metastasis and measure tumor response to chemotherapy.

5.3.6 *Selective Expression of Biomarkers by Cancer Compared with Normal Tissues*

NCI scientists have used archival samples inclusive of normal tissues of various lineages and benign or malignant tumors (predominantly colon, melanoma, ovarian, and esophageal cancers) to study cancer biomarkers (Basil et al. 2006). All samples were processed identically and cohybridized with an identical reference RNA source to a custom-made cDNA array platform. The database was split into training and comparable prediction sets. Leave-one-out cross-validation and gene pairing analysis identified putative cancer biomarkers overexpressed by malignant lesions independent of tissue of derivation. In particular, seven gene pairs were identified with high predictive power (87 %) in segregating malignant from benign lesions. Receiver operator characteristic curves based on the same genes could segregate malignant from benign tissues with 94 % accuracy. The relevance of this study rests on the identification of a restricted number of biomarkers ubiquitously expressed by cancers of distinct histology. This has not been done before. These biomarkers could be used broadly to increase the sensitivity and accuracy of cancer staging and early detection of locoregional or systemic recurrence. Their selective expression by cancerous compared with paired normal tissues suggests an association with the oncogenic process resulting in stable expression during disease progression when the presently used differentiation markers are unreliable.

5.3.7 *Ultrasound Radiation to Enhance Release of a Tumor Biomarker*

In studies on tumor-bearing mice, application of low-frequency ultrasound to tumor cells has been demonstrated to enhance the release of CEA, a biomarker of cancer, which can be measured in the blood (D'Souza et al. 2009). It was further established that this release is specific to the direct application of the ultrasound to the tumor, enabling a method for localization of biomarker production. Blood concentrations of that biomarker rose significantly only when ultrasound energy was directed to tumor sites but not when the ultrasound beam was focused on non-tumor-bearing tissues. This work will enable the detection of cancer in the presymptomatic stage using a relatively simple and noninvasive strategy. Future work using image-guided focused ultrasound to radiate tumors with ultrasound should help to bring together the currently separate fields of in vitro diagnostics and in vivo imaging and facilitate the development of personalized medicine. There are no significant regulatory impediments to the integration of this method into clinical practice, as ultrasound is already widely used in the clinic. It will be necessary to optimize the technique for use in humans, and it will not work for all tumor types, e.g., lung or bone marrow cancers, because ultrasound is impeded by bony structures and air-filled zones in the body. The proof of principle has been established for a single biomarker, CEA, and other biomarkers may prove more difficult to measure by this approach.

5.3.8 In Vivo Imaging of Cancer Biomarkers

Various "omic" approaches are providing comprehensive "snapshots" of biomarkers of cancer, but imaging can take this information a step further, showing the activity of these biomarkers in vivo and how their location changes over time. Advances in experimental and clinical imaging are likely to improve the understanding of cancer at the systems level and, ultimately, should enable doctors not only to locate tumors but also to assess the activity of the biological processes within these tumors and to provide "on the spot" treatment (Weissleder and Pittet 2008). Several technologies described earlier can be used for in vivo imaging of biomarkers in cancer. The best known of these are computer tomography (CT), MRI, and PET.

5.3.8.1 Computer Tomography

CT is used not only for diagnosis but also for measurement of volume of a tumor. 3D tumor imaging is better as a "surrogate endpoint" of measuring drug responsiveness rather than the unidimensional measurements currently used. Tumor reduction assessed by high-resolution CT has been used successfully as an endpoint in a phase II trial of pazopanib, an oral angiogenesis inhibitor targeting VEGFR, PDGFR, and c-kit in patients with stage I–II non small cell lung cancer (NSCLC). CT, however, measures only the size but not metabolic and other changes in cancer at molecular level in response to treatment that may be better indicators of response.

5.3.8.2 Optical Systems for In Vivo Molecular Imaging of Cancer

Some molecular specific contrast agents for molecular imaging are based on gold NPs, which are attached to probe molecules with high affinity for specific cellular biomarkers. The application of gold bioconjugates for vital imaging of precancers has been shown by using cancer cell suspensions, 3D cell cultures, and neoplastic fresh cervical biopsies. Gold conjugates as contrast agents have potential to extend the ability of vital reflectance microscopies for in vivo molecular imaging. They can potentially enable combined screening, detection, and therapy of disease using inexpensive imaging systems; such tools could allow mass screening of diseases such as cancer in resource-poor settings.

In order to use such optical systems to image molecular features of cancer, it will be necessary to deliver sufficient contrast agent to tissue so that a reasonable signal-to-noise ratio can be obtained. Before contrast agents can be used in human subjects, extensive animal studies must be carried out to evaluate any potential toxicity of these contrast agents and delivery formulations. In spite of these concerns, these contrast agents and imaging systems have the potential to significantly impact current clinical practice.

5.3.8.3 Positron Emission Tomography

During the past decade, PET has been increasingly developed for imaging and quantifying molecular mechanisms in oncology. The technique uses radionuclides to label molecules, which can then be imaged in man. PET with 18F-fluorodeoxyglucose (FDG) as a radioactive tracer for glucose metabolism is currently an effective and highly utilized tool for the diagnosis and management of cancer. Growing tumors consume glucose and show up as bright spots on PET that can disappear a week after a patient begins chemotherapy, signaling possible remission. Waiting for demonstrable tumor shrinkage on computed tomography scans takes another 6 months. Seeing whether a patient actually lives longer could take years. Usually, changes in PET results are not dramatic enough to be seen by the naked eye, but the signal must be interpreted through a series of calculations gauging the concentration of a radioactive tracer.

Continuing technological improvements in imaging, including higher resolution, better attenuation correction, and multimodality image registration, will further improve the efficacy of this method. The most significant improvements will come from the wide variety of tracers now being developed to image other metabolic pathways and to identify cancer by specific biochemical, physiological, and genetic characteristics. Developments in PET mean that a wider range of molecules can now be labeled with isotopes and old and new molecular targets for anticancer therapy can be probed, imaged, and quantified in vivo in man. The inherent sensitivity and specificity of PET is unrivalled because it can image molecular interactions and pathways, providing quantitative kinetic information down to the subpicomolar level. Molecular imaging has the potential to assist in the optimization of molecular-based targeted therapies in cancer and to investigate the function of the genome.

5.3.8.4 Imaging of Tumor Oxygenation and Microvascular Permeability by MRI

Increased vascular permeability and disturbance of blood flow impairs delivery of oxygen and drugs to tumors leading to treatment resistance. Tissue oxygen concentration and microvascular permeability can be visualized simultaneously by using Overhauser-enhanced MRI (a hyperpolarized 1H-MRI) and OX63, an oxygen-sensitive contrast agent (Matsumoto et al. 2009). Application of this method for imaging murine tumors showed that tumor regions with high vascular permeability were also hypoxic with an inverse correlation between tumor vascular leakage and oxygen concentration. This imaging technique may be useful for the assessment of changes in vascular permeability and oxygenation of tumor in response to chemotherapy, radiotherapy, or antiangiogenic treatment.

5.3.8.5 Xenon-Enhanced MRI

Researchers at the University of Pennsylvania (Philadelphia, PA) are extending the capabilities of MRI for monitoring multiple cancer biomarkers simultaneously using the xenon as an imaging agent. Encapsulation of a single atom of xenon within a cage made of cryptophane provides a sensitive reporter of changes outside the cage. When the cage encounters a specific cancer protein, the xenon molecule emits a telltale signal that can be tracked by MRI. Based on this principle, new biosensors are being generated that will identify biomarkers associated with cancers of the lungs, brain, and pancreas. Thus, MRI can be used to detect aberrant proteins that cause cancer in humans before the actual formation of a tumor.

5.3.9 Kallikrein Gene Family and Cancer Biomarkers

The tissue kallikreins are serine proteases that are encoded by highly conserved multigene family clusters in rodents and humans. In vitro biochemical studies show that some kallikreins can auto-activate and others can activate each other, suggesting that the kallikreins may participate in an enzymatic cascade similar to that of the coagulation cascade (Clements et al. 2004). Human tissue kallikrein genes, located on the long arm of chromosome 19, are a subgroup of the serine protease family of proteolytic enzymes. Human kallikrein locus has now been extended and includes 15 tandemly located genes (Obiezu and Diamandis 2005). These genes, and their protein products, share a high degree of homology and are expressed in a wide array of tissues, mainly those that are under steroid hormone control. Kallikreins (KLK4–KLK15), which have been associated with several types of cancer, are emerging biomarkers for ovarian, breast, prostate, and testicular cancers. New evidence raises the possibility that some kallikreins are directly involved with cancer progression.

5.3.10 Detection of CTCs as Biomarkers of Cancer

There is need for noninvasive diagnostic method to confirm the presence of cancer. Blood samples have been analyzed for CTCs as biomarkers by using nucleic acid methods to isolate tumor-associated or tumor-specific mRNA. Detection of extremely low concentrations of rare cancer cells in the blood is still a challenge. The preferred method of detection, automated digital microscopy (ADM), is too slow to scan the large substrate areas. Fiber-optic array scanning technology (FAST) applies laser-printing techniques to the rare-cell detection problem. With FAST cytometry, laser-printing optics is used to excite 300,000 cells per seconds, and

emission is collected in an extremely wide field of view, enabling a 500-fold speedup over ADM with comparable sensitivity and superior specificity. The combination of FAST enrichment and ADM imaging has the performance required for reliable detection of early-stage cancer in blood.

The CellTracks® AutoPrep® System (Immunicon Corporation) is an automated sample preparation system for immunomagnetic cell capture and fluorescence staining of rare cells. It is used with the company's reagent kits to automate and standardize the isolation of CTCs. The CellTracks® Analyzer II is a semiautomated fluorescence microscope that is used to count and characterize the immunomagnetically selected cells based on the fluorescence signals of the cells. Immunicon's technology for the quantification and characterization of rare cells is being used in drug development trials as efficacy biomarkers, for risk stratification and to monitor expression levels of proteins associated with targeted therapy. Detection of CTCs using immunomagnetics before initiation of first-line therapy in patients with MBC is highly predictive of progression-free survival and overall survival. Increased numbers of circulating endothelial cells (CECs) are observed in peripheral blood of cancer patients, which may contribute to tumor growth through the process of angiogenesis. Characterization of these cells by study of gene expression profiles of immunomagnetically enriched CECs may provide biomarkers to evaluate treatment efficacy. This technology can aid in appropriate patient stratification and design of tailored treatments.

CellSearch (Veridex), based upon immunomagnetic technologies and constituted by magnetic NPs coated with anti-EpCAM antibodies, currently represents one of the best systems of CTCs detection. It is approved by the FDA. Prior to the start of a new line of chemotherapy, CTC identification through CellSearch has prognostic value in breast, prostate, and CRC (O'Flaherty et al. 2012).

Although extremely rare, CTCs represent a potential alternative to invasive biopsies as a source of tumor tissue for the detection, characterization, and monitoring of non-hematological cancers. The ability to identify, isolate, propagate, and molecularly characterize CTC subpopulations could further the discovery of cancer stem cell (CSC) biomarkers and expand the understanding of the biology of metastasis. Current strategies for isolating CTCs are limited to complex analytical approaches that generate very low yield and purity. A unique microfluidic platform (the "CTC-Chip") is capable of efficient and selective separation of viable CTCs from peripheral whole-blood samples, mediated by the interaction of target CTCs with antibody (EpCAM)-coated microposts under precisely controlled laminar flow conditions, and without requisite pre-labeling or processing of samples (Nagrath et al. 2007). The CTC-Chip has successfully identified CTCs in the peripheral blood of patients with metastatic lung, prostate, pancreatic, breast, and colon cancers in 99 % of samples. In the near future, refinements of techniques for isolation of tumor cells in the blood and their characterization may emerge as a powerful diagnostic tool, facilitating cytogenetic analysis, early detection of cancer, localization of tumor, therapy selection, and determination of chemoresistance.

5.4 Applications of Cancer Biomarkers

The main clinical applications of cancer biomarkers are:

- Classification of tumors
- Prognosis of disease progression
- Prediction of response to therapy
- Monitoring of response to therapy

Some of these are described in Chap. 16 (Personalized Cancer Therapy).

5.4.1 Classification of Cancer Using Proteomic Biomarkers

The use of rapid, high-throughput MS-based fingerprints of peptides and proteins may prove to be valuable for new molecular classification of human tumors and disease stages. Coupled with LCM, high-density protein arrays and antibody arrays will have a substantial impact on proteomic profiling of human cancers.

5.4.2 Use of Biomarkers for Early Detection of Cancer

Although plasma tumor biomarkers are widely used clinically for monitoring response to therapy and detecting cancer recurrence, only a limited number of them have been used effectively for the early detection of cancer. A review of the use of cancer biomarkers in the USA shows that only PSA, cancer antigen 125, and alpha-fetoprotein have been clinically used for the early detection of prostate, ovarian, and liver cancers, respectively (Meany et al. 2009). Few plasma tumor biomarkers have been used effectively for the early detection of cancer, mainly because of their limited sensitivity and/or specificity. Several approaches are being developed to improve the clinical performance of tumor biomarkers for the early detection of cancer.

5.4.3 Applications of Biomarkers for Cancer Diagnosis

5.4.3.1 Methylated DNA Sequences as Cancer Biomarkers

To identify and overcome barriers in the application of methylated genes as cancer biomarkers and to promote validation studies of these biomarkers, the NCI Early Detection Research Network (EDRN) joined forces with the National Institute of Standards and Technology (NIST) to conduct a workshop on Standards and Metrology for Cancer Detection and Diagnostics focusing upon DNA methylation

The results of the Workshop have been published (Kagan et al. 2007). The objectives of the workshop were:

- To evaluate methods and standards for robust, sensitive, and preferentially quantitative measurements of DNA methylation in clinical specimens
- To evaluate demands stemming from different types of specimens (e.g., tissue versus biological fluids)
- To identify and evaluate variables (e.g., amount of DNA template) influencing the robustness of the particular assay
- To evaluate the need, and develop recommendations, for Standard Reference Materials for the discovery and validation of methylated DNA biomarkers (including cross-validation between laboratories and platforms)
- To evaluate the need and make recommendations regarding the necessity to establish a common collection of data standards that can be used to transmit cancer-related clinical research data among investigators, clinicians, and regulators

It was clear from this workshop that one standard cannot be developed for addressing all the applications for methylation in the basic and translational research fields as well as the clinical testing. The best technology depends on the question being asked. However, the development of standard assays will require standard specimens for clinical comparison. The most straightforward set of specimens are tumor cell lines, which can be regenerated and provide an unlimited source of DNA. Tumor tissue and adjacent tissue from a cancer that is common and always resected, such as colon cancer, could be a second valuable standard for assay validation. It also is clear that there is a pressing need for perhaps unexciting, but important, studies to determine the optimal parameters for choice, storage, and preparation of clinical specimen for DNA isolation, bisulfite modification, and technology controls. The conclusions of this workshop about the desirable characteristics of methylated DNA sequences as clinical biomarkers were as follows:

- Reproducible, preferentially quantitative measurement is important in all clinical applications.
- Absolute methylation measurement (% methylation at individual sites) is more amenable to precise quantitation.
- Individual gene methylation measurement will likely be clinically useful in cancer detection, diagnosis and prognosis, and classification and possibly in risk assessment.
- The performance of a biomarker is highly dependent on the choice of methylation detection method.

Choices of technology recommendations were as follows:

- Bisulfite sequencing is optimal for the analysis of CpG island methylation of new genes.
- Pyrosequencing is optimal for quantitation of individual CpG sites.
- Quantitative MSP is optimal for sensitive detection of methylated alleles.

Standardization issues and recommendations were as follows:

- Different genes used in detection assays: establish optimal gene panel by interlaboratory testing.
- Different area of promoter of same gene: establish optimal gene panel by interlaboratory testing.
- Different technology used for analysis of methylation status: establish by interlaboratory testing of aliquots from universal standard.
- Different reference or controls used with same technology: establish by interlaboratory testing of aliquots from universal standard.

5.4.3.2 MicroRNA Expression Profiling for Diagnosis of Human Cancers

More than 200 microRNAs (miRNAs) are known. Although their function is not well understood, they control gene activity and play a major role in the development of human cancers. Bead-based flow cytometric expression profiling of miRNAs in samples from multiple human cancers has shown distinctive miRNA fingerprints (Lu et al. 2005a). Generally there was downregulation of miRNAs in tumors compared with normal tissues. The miRNA profiles reflect the developmental lineage and differentiation state of the tumors and enabled successful classification of poorly differentiated tumors, whereas mRNA profiles were highly inaccurate when applied to the same samples. These findings highlight the potential of miRNA profiling in cancer diagnosis and to select the most appropriate treatment.

5.4.3.3 MUC4 as a Diagnostic Biomarker in Cancer

Mucins are high molecular mass glycoproteins whose role in diagnosis, prognosis, and therapy is being increasingly recognized owing to their altered expression in a variety of carcinomas. MUC4, a membrane-bound mucin encoded by a gene located on chromosome locus 3q29, is aberrantly expressed in several cancers including those of the bile duct, breast, colon, esophagus, ovary, lung, prostate, stomach, and pancreas. MUC4 expression pattern has potential use in the diagnosis and prognosis of various cancers (Chakraborty et al. 2008). MUC4 expression is a specific biomarker of epithelial tumors, and its expression correlates positively with the degree of differentiation in several cancers. MUC4 has emerged as a specific biomarker of dysplasia, being expressed in the earliest dysplastic lesions preceding several malignancies, including pancreatic cancer. The presence of MUC4-specific antibodies in the serum and of the transcript in peripheral blood mononuclear cells of cancer patients may lead to a biomarker-based test for bedside application in high-risk individuals and those with established cancer.

5.4.4 Applications of Biomarkers for Cancer Diagnosis and Therapy

The effectiveness of the Bcr–Abl kinase inhibitor imatinib (Novartis' Gleevec) in chronic myeloid leukemia (CML) and in a subset of patients with acute lymphoblastic leukemia (ALL) reduces with advancing disease and/or the development of resistance to imatinib. AMN-107 (Novartis' Tasigna®) inhibits the proliferation of hematopoietic cells expressing the mutants in Ph+ CML and ALL and is also effective against several imatinib-resistant Bcr–Abl mutants. This drug is combined with a battery of tests to define which patients should receive it.

More and more new drugs in oncology are being pursued with parallel development of a diagnostic test. As genotyping of drug-metabolizing enzymes becomes more widespread in the future, more changes are expected in drug labels.

With new targeted therapies for cancer, the role of biomarkers is increasingly promising, suggesting an integrated approach using the genetic makeup of the tumor and the genotype of the patient for treatment selection and patient management. Biomarkers can aid in patient stratification (risk assessment), in treatment response identification (surrogate markers), or in differential diagnosis (identifying individuals who are likely to respond to specific drugs). To be clinically useful, a marker must favorably affect clinical outcomes such as decreased toxicity, increased overall and/or disease-free survival, or improved quality of life. Once the methods for assessment of the biomarker are established and the initial results show promise with regard to the predictive ability of a marker, it may be possible to achieve the goal of "predictive oncology."

Variation in the PI3K gene could be a key biomarker for use as a companion diagnostic with certain cancer treatments. Several studies suggest that mutations in the PI3K oncogene are predictive for the success of certain treatments of patients suffering from lung, breast, colorectal, and other cancers. QIAGEN has an active PI3K assay development and partnering program with pharmaceutical companies to develop and market tests based on this for new cancer drug candidates.

5.4.4.1 Peptide-Based Agents for Targeting Cancer Biomarkers

Small peptide-based agents have attracted wide interest as cancer-targeting agents for diagnostic imaging and targeted therapy. Efforts are being made to develop new high-affinity and high-specificity peptidomimetic or small-molecule ligands against cancer cell surface receptors. A high-affinity peptidomimetic ligand (LLP2A) against α4β1 integrin has been identified using both diverse and highly focused one-bead, one-compound combinatorial peptidomimetic libraries in conjunction with high-stringency screening (Peng et al. 2006). LLP2A can be used to image α4β1-expressing lymphomas with high sensitivity and specificity when conjugated to a near infrared fluorescent dye in a mouse xenograft model. Thus, LLP2A provides an

important tool for noninvasive monitoring of α4β1 expression and activity during tumor progression, and it shows great potential as an imaging and therapeutic agent for α4β1-positive tumors.

5.4.5 Biomarkers for Assessing Efficacy of Cancer Therapy

The high incidence of resistance to DNA-damaging chemotherapeutic drugs and severe side effects of chemotherapy have led to a search for biomarkers able to predict which patients are most likely to respond to therapy.

5.4.5.1 ERCC1–XPF Expression as a Biomarker of Response to Chemotherapy

ERCC1–XPF nuclease is required for nucleotide excision repair of DNA damage by cisplatin and related drugs, which are widely used in the treatment of cancer. The levels of ERCC1–XPF in a tumor could indicate whether it will be sensitive or resistant to a certain chemotherapeutic agent. Although several commercially available antibodies are suitable for immunodetection of ERCC1–XPF in some applications, only a select subset is appropriate for detection of this repair complex in fixed specimens. A study provides reliable tools for clinicians to measure the enzyme ERCC1–XPF as a biomarker in clinical specimens that could help stratify patients according to cancer risk, response to treatment, and overall prognosis (Bhagwat et al. 2009).

5.4.5.2 P53 Expression Level as Biomarker of Efficacy of Cancer Gene Therapy

Advexin (Introgen Therapeutics) is a gene-based drug, injected directly into tumors, which uses an adenoviral vector to deliver the wild-type p53 gene to tumor cells. Patients with advanced squamous cell carcinoma of the head and neck cancer, whose pretreatment tumor samples overexpressed p53, were significantly more likely to respond to Advexin therapy than those whose tumor showed little p53 protein. FDA has agreed to the use of Introgen's p53 molecular biomarkers in the analysis of Advexin clinical data used in support of submissions for approval. In updated data from phase II clinical trials, the predictive abnormal p53 biomarker was associated with a statistically significant increase in tumor responses to Advexin therapy. A reduction in tumor size was observed in 40 % of patients with the abnormal p53 biomarker compared to none (0 %) of the patients with p53 normal tumors (Nemunaitis et al. 2009). This makes p53 the first predictive biomarker test for a gene-based drug. Not only is this a way to predict if the gene therapy is likely to succeed, the patients for which it does work are the most difficult ones to treat. Accumulation of p53 corresponds with a poor response to traditional therapies such

as radiation and chemotherapy, as well as lower survival and a shorter time to disease progression. Advexin has also achieved 100 % response when combined with chemotherapy to treat locally advanced breast cancer and a 69 % response when used with radiation to treat NSCLC.

5.4.6 Biomarkers of Angiogenesis for Developing Antiangiogenic Therapy

Angiogenesis, the formation of new blood vessels, is associated with normal physiological processes such as wound healing, ovulation, or menstruation as well as with many diseases, such as solid tumors. Recent findings about the pathomechanisms of tumor angiogenesis have led to new therapeutic options in the treatment of malignant tumors. During the development of antiangiogenic drugs, reports ranged from curing cancer to completely ineffective drugs. Some antiangiogenic agents have been approved and others are still in development. Many antiangiogenic drugs may encounter problems during clinical trials because they cannot reduce tumor size rapidly like chemotherapies can. This is another reason why biomarkers are needed for determining the effectiveness of antiangiogenic drugs in an early stage. Currently, there is a need to identify biomarkers that can both indicate biological activity and predict efficacy at the molecular level for antiangiogenesis drugs which are anticipated to result in tumor stasis rather than regression.

5.4.6.1 Biomarkers of Response to Antiangiogenic Agents

In order to identify biomarkers of response, athymic mice bearing L2987 human tumor xenografts were treated with the antiangiogenic agent brivanib alaninate (Bristol-Myers Squibb Co.), which is currently under clinical evaluation (Ayers et al. 2007). This is an orally available and selective tyrosine kinase inhibitor that targets the key angiogenesis receptors VEGFR-2 and FGFR-1. For these studies, tumor samples were collected from the xenografts, and RNA was extracted for gene expression profiling on Affymetrix 430A mouse GeneChips. Statistical analysis was done using a defined set of genes identified to be coexpressed with VEGFR-2 from a clinical tumor gene expression profiling database and between tumor samples isolated from brivanib alaninate-treated and untreated mice. Tyrosine kinase receptor 1 (Tie-1), collagen type IVα1 (Col4a1), complement component 1, q subcomponent receptor 1 (C1qr1), angiotensin receptor-like 1 (Agtrl1), and vascular endothelial-cadherin (Cdh5) were all identified to be significantly modulated by treatment with brivanib alaninate. These genes, which may be potentially useful as biomarkers of brivanib alaninate activity, were further studied at the protein level in human colon tumor xenograft models, HCT116 and GEO, using IHC-based approaches.

5.4.6.2 Circulating Endothelial Cells as Targets for Antiangiogenic Drugs

Previous studies have shown that the blood circulation of cancer patients has an abnormally high number of endothelial cells, which help construct blood vessels including those that feed the cancerous tumor. In addition to growing them directly from nearby blood vessels, tumors can also signal the body's bone marrow to boost the supply of endothelial cells in the blood circulation. Antiangiogenic drugs might combat cancer by preventing immature cells in the bone marrow from developing into endothelial cells. According to NCI researchers, if an antiangiogenic drug is successfully starving a cancer patient's tumor to death, the number of CECs in the individual's bloodstream will decrease, thus providing a potential biomarker for gauging the medication's effectiveness.

Antiangiogenic drugs inhibit blood vessel development at the tumor by killing the endothelial cells lining tumor blood vessels and/or cutting off the supply of endothelial cells from bone marrow. These drugs are typically paired with chemotherapy agents. Unlike antiangiogenic drugs, chemotherapy agents directly attack tumor cells, and a reliable therapeutic biomarker for evaluating these agents is whether there are fewer cells in the tumor, or more, or just the same amount as before the treatment. Some chemotherapy drugs also have the benefit of being toxic to endothelial cells, providing a possible second biomarker for those agents.

5.4.6.3 Imaging Biomarkers for Evaluation of Antiangiogenic Agents

Some of the challenges posed by evaluation by imaging of angiogenesis inhibitors in phase I/II clinical trials. Because they reduce tumor growth or prevent metastases through primarily cytostatic modes of action—selectively inhibiting membrane receptors, cell cycle regulators, or other signaling pathways—conventional endpoints based on reduction in tumor size may be inadequate for evaluating clinical response. Alternative imaging biomarkers of angiogenesis are being sought, which can serve as early indicators of drug activity in clinical trials and may facilitate early pharmacodynamic assessments by speeding up the go/no-go decision-making process. Dynamic contrast-enhanced-magnetic resonance imaging (DCE-MRI) is now frequently used in early clinical trial assessment of antiangiogenic and vascular disrupting compounds. Evidence of drug efficacy and dose-dependent response has been demonstrated by use of DCE-MRI with some angiogenesis inhibitors (O'Connor et al. 2007). Validation against histopathology biomarkers such as microvascular density is problematic in DCE-MRI, where micrometer scale biopsy changes must be compared against voxel resolution in millimeters. Nonetheless, histopathology validation is important and can substantiate the use of a biomarker in phase I/II trials. Both animal models and clinical studies are likely to be required to achieve comprehensive validation.

In contrast to identifying the maximal tolerable dose, determination of the optimal biological dose, i.e., reaching biological activity at lower doses, has become the main target in the early development of antiangiogenic agents. This has been evaluated by

different biomarker techniques. As a new standard in antitumor treatment, a better understanding of imaging in the treatment monitoring for antiangiogenic agents is important. Studies of tumor angiogenesis by tissue sampling rely on invasive procedures, adequate sampling, and painstaking estimation of histological microvessel density. Attempts to develop wound healing assays to correlate angiogenesis in wounds with angiogenesis in tumor have been made but are still considered invasive and correlation of healthy with malignant tissue is still of limited validity. Several soluble biomarkers of tumor angiogenesis have been detected in various malignant diseases and have been evaluated for their use as surrogate markers in tumor angiogenesis. Soluble biomarkers were further investigated for use as imaging tools. Combination of biomarkers and imaging techniques has become an important method for developing anticancer drugs, an individual patient's response, and monitoring of success of therapy at an early stage. Thus, time-consuming delays due to anatomy-based restaging procedures can be avoided. Characterization of soluble biomarkers can be combined with different imaging techniques such as ultrasound, CT, MRI, and PET.

5.4.6.4 Tumor Endothelial Markers

Lack of cancer-specific endothelial markers has hindered the development of cancer therapies targeted against angiogenesis. Although the ability of Avastin to prolong survival in a phase III clinical trial of human CRC has established the validity of the antiangiogenic approach, realization of the full potential of a vascular targeting strategy may require the exploitation of molecules which are highly restricted in expression to tumor endothelium.

Specific biomarkers can be used to identify tumor angiogenesis, as distinct from normal physiological angiogenesis. Several such biomarkers have been identified by using comparative gene expression analysis on various normal and tumor endothelial cells. Technological advances in cellular fractionation and genomics enabled the identification of several markers preferentially expressed on human tumor endothelium. Tumor endothelial markers (TEMs) have the potential as new targets for cancer therapy. Studies of these TEMs are expected to aid our understanding of angiogenesis and could lead to the development of new imaging and diagnostic agents for cancer. For example, the identification of TEM8 as the anthrax toxin receptor and the successful targeting of this receptor in preclinical tumor models make this molecule a particularly attractive candidate for future vascular targeting studies. Some of the secreted TEMs can serve as surrogate markers in the determination of the optimum biological dose for the current antiangiogenic drugs in clinical trials.

5.4.6.5 VEGF Signaling Inhibitors as Biomarkers

A systematic review using PubMed, MEDLINE, and American Society of Clinical Oncologist databases was conducted for articles (including abstracts) presented

from 2007 to 2009 to compare new small-molecule tyrosine kinase inhibitors with VEGF receptor as one of their targets (Wood et al. 2009). Factors considered included mode of action (targets), toxicity, and usefulness of biomarker data. Search terms included "angiogenesis inhibitors," "tyrosine kinase inhibitors," "VEGF" and "biomarkers." Nine compounds were selected for detailed comparison. The toxicity profiles of the compounds were similar. Many exposure biomarkers were identified that helped to determine the dose and scheduling of these compounds in clinical trials. Progress has also been made in identifying potential efficacy and predictive biomarkers for these new agents; however, these are yet to be validated.

5.4.6.6 VEGF-PET Imaging for Analysis of Angiogenic Changes Within a Tumor

Noninvasive imaging of angiogenesis could ease the optimization of antiangiogenesis treatments for cancer. A study has evaluated the role of VEGF-PET as a biomarker of dynamic angiogenic changes in tumors following treatment with the kinase inhibitor sunitinib (Nagengast et al. 2010). The effects of sunitinib treatment and withdrawal on the tumor were investigated using the new VEGF-PET tracer ^{89}Zr-ranibizumab as well as ^{18}F-FDG PET and ^{15}O-water PET in mouse xenograft models of human cancer. The imaging results were compared with tumor growth, VEGF plasma levels, and immunohistologic analyses. In contrast to ^{18}F-FDG and ^{15}O-water PET, VEGF-PET demonstrated dynamic changes during sunitinib treatment within the tumor with a strong decline in signal in the tumor center and only minimal reduction in the tumor rim, with a pronounced rebound after sunitinib discontinuation. VEGF-PET results corresponded with tumor growth and immunohistochemical vascular and tumor biomarkers. These findings highlight the strengths of VEGF-PET imaging to enable serial analysis of angiogenic changes in different areas within a tumor.

5.4.7 Biomarkers of Prognosis in Cancer Treatment

Various biomarkers of prognosis in cancer treatment have been investigated, and some novel ones are shown in Table 5.3. Prognosis is considered in more detail along with biomarkers of individual cancers.

5.4.8 Biomarkers for Monitoring Cancer Therapy

Tumor biomarkers that are measured during postoperative surveillance are also frequently used for monitoring patients with advanced cancer receiving systemic therapy. Examples are CEA for CRC, CA 15-3 for breast cancer, and alpha fetoprotein

Table 5.3 Novel biomarkers of prognosis in cancer treatment

Biomarker	Role in cancer	Findings in cancer	Prognostic significance
PI3K, an enzyme that helps control cell growth	Mutations in PIK3CA, the gene encoding the catalytic subunit of PI3K, activate the PI3K/AKT/mTOR pathway in cancer cells	PIK3CA mutations are frequent in cancers of endometrium, ovaries, and breast	In phase I clinical trials, 40 % of patients with mutations in PI3K gene responded to treatment targeting PI3K signaling
CYP2D6, role in metabolizing several drugs	Polymorphisms of the gene may interfere with action of anticancer drugs	Polymorphisms of CYP2D6 and coadministration of drugs that inhibit CYP2D6 reduce plasma levels of endoxifen, the active metabolite of tamoxifen, in patients being treated for breast cancer	Patients who only took CYP2D6-inhibiting medications in conjunction with tamoxifen had worse time to progression and worse overall survival compared to patients who did not take CYP2D6 inhibitors
TIMP-1, tissue inhibitor of matrix metalloproteinase 1	TIMP-1 is overexpressed in many cancers	TIMP-1 is associated with poor outcome in renal cell carcinoma (RCC)	Sorafenib (a multikinase inhibitor) phase III TARGET trial showed that TIMP-1 is an independent prognostic biomarker for survival in RCC
Trim62, a novel protein biomarker	Trim62 regulates p27 stability but also its localization in HER2 positive breast cancers. p27 in the nucleus functions as a tumor suppressor but, when in the cytoplasm, it may enhance metastasis	Trim62 is responsible for the elevated cytoplasmic p27 in HER2 positive breast cancer tumors	Trim62 could be a potential biomarker to predict patient response to anti-HER2 therapeutics such as lapatinib

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(AFP) for hepatocellular cancer. Usually decreasing levels of biomarkers following the initiation of therapy correlates with tumor regression, and increasing levels predict progressive disease. Tumor biomarkers, however, should not be the sole criteria for assessing response to therapy. A caveat in the use of biomarkers for monitoring therapy in patients with advanced cancer is that transient increases or spikes may occur within the first few weeks of start of therapy, which appear to be due to tumor cell necrosis or apoptosis in response to the initial treatment with chemotherapy (Duffy 2006). Such transient increases have not yet been reported with biological therapies such as therapeutic antibodies, e.g., Herceptin, cetuximab, and panitumumab.

5.4.9 Biomarkers of Drug Resistance in Cancer

Human cancers are mostly found to be resistant to therapy at the time of drug presentation (primary responses), tumors being intrinsically drug resistant (innate or de novo drug resistance). Only a few become resistant after an initial response (acquired responses), the tumors developing resistance to chemotherapy during treatment (acquired drug resistance). In the latter group, a tumor cell may express drug resistance by combining several distinct mechanisms induced by its exposure to various drugs. In the former group, however, this is unlikely to be the case.

Pharmacogenetic and pharmacogenomic studies of the relationship between individual variations and drug response rates reveal that genetic polymorphisms of specific genes are associated with clinical outcomes in patients treated through chemotherapy, and amplification of genes encoding drug targets or transporters alters the sensitivity of cancer cells to a particular chemotherapy. LOH at specific regions of chromosomes has been identified in specific cancers, but its effect on treatment outcome remains controversial.

5.4.9.1 A Systems Approach to Biomarkers of Innate Drug Resistance

In a biological systems approach to understand innate CRC tumor responses to a FOLFIRI-combined chemotherapy of irinotecan (CPT-11) plus 5-FU/FA, gene expression patterns obtained with microarrays were compared between clinical samples from colon tumors and liver metastases collected from CRC patients prior to drug exposure (Grauden et al. 2006). Data collected from a biological systems perspective into global and interconnected molecular networks highlight the molecular mechanisms that may anticipate resistance in CRC patients prior to their exposure to drugs. The information generated in this study might also provide new biomarkers for prediction of the chemosensitive and chemoresistant states to the combined chemotherapy in newly diagnosed CRC patients, enabling therapeutic adjustment. Further integration with data collected at the genomic level through mutation analysis, at the level of the entire transcriptome by complementary comprehensive methods such as massive parallel signature sequencing, at the proteome level with emerging global technologies such as ICAT coupled with MS, and at the metabolome level by MS or NMR should provide the

basis for designing reliable predictive biomarkers and deciphering the molecular pathways involved in drug responses.

5.4.9.2 Epithelial Membrane Protein-1 as a Biomarker of Gefitinib Resistance

Gefitinib is a small-molecule inhibitor that competes for the ATP-binding site on epithelial growth factor receptor (EGFR) and has been approved for patients with advanced lung cancers. Treatment with gefitinib has resulted in clinical benefit in patients, and, recently, heterozygous somatic mutations within the EGFR catalytic domain have been identified as a clinical correlate to objective response to gefitinib. However, clinical resistance to gefitinib limits the utility of this therapeutic to a fraction of patients, and objective clinical responses are rare. Epithelial membrane protein-1 (EMP-1), an adhesion molecule, has been identified as a surface biomarker whose expression correlates with acquisition of gefitinib resistance (Jain et al. 2005). EMP-1 expression further correlates with lack of complete or partial response to gefitinib in lung cancer patient samples as well as clinical progression to secondary gefitinib resistance. EMP-1 expression and acquisition of gefitinib clinical resistance is independent of gefitinib-sensitizing EGFR somatic mutations. There is a probable cross talk between this molecule and the EGFR signaling pathway.

5.4.9.3 Methylation Biomarkers of Drug Resistance in Cancer

The association of the methylation status of DNA repair genes such as O^6-methylguanine-DNA methyltransferase (MGMT) and MLH1 illustrates the two main mechanisms of response to DNA-damaging agents. Loss of methylation of MGMT, and the subsequent increase in gene expression, leads to a reduction in response to alkylating agents as a result of enhanced repair of drug-induced DNA damage. Conversely, the increase in methylation of MLH1 and its resulting loss of expression have been consistently observed in drug-resistant tumor cells. MLH1 encodes a mismatch repair enzyme activated in response to DNA damage; activation of MLH1 also induces apoptosis of tumor cells, and thus loss of its expression leads to resistance to DNA-damaging agents. Other methylation-regulated genes that could serve as biomarkers in cancer therapy include drug transporters, genes involved in microtubule formation and stability, and genes related to hormonal therapy response. These methylation markers have potential applications for disease prognosis, treatment response prediction, and the development of novel treatment strategies for cancer.

5.4.9.4 STAT3 and Resistance to Cisplatin

STAT3 (signal transducer and activator of transcription 3) seems to play crucial roles in cell proliferation and survival, angiogenesis, tumor-promoting inflammation, and suppression of antitumor host immune response in the tumor microenvironment.

STAT3 is central to determining the type of inflammation that can either promote or inhibit tumor development; direct inhibition of STAT3 may represent a promising therapeutic target to reprogram tumor-promoting inflammation into tumor-suppressing inflammation (Kato 2011).

STAT3 is elevated in ~82 % of head and neck cancers and has been associated with cisplatin resistance. STAT3 inhibitors such as FLLL32 (a compound based on curcumin) may be useful adjuvants to cisplatin to overcome drug resistance (Abuzeid et al. 2011).

5.4.10 Biomarkers of Radiation Therapy for Cancer

Radiation therapy is used to treat half of all cancer patients. Response to radiation therapy varies widely among patients. A study has explored the merit of proteomic profiling strategies in patients with cancer before and during radiotherapy in an effort to discover clinical biomarkers of radiation exposure (Menard et al. 2006). High-resolution SELDI-TOF MS was used to generate high-throughput proteomic profiles of unfractionated serum samples using an immobilized metal ion-affinity chromatography nickel-affinity chip surface. Resultant proteomic profiles were analyzed for unique biomarker signatures using supervised classification techniques. MS-based protein identification was then done on pooled sera in an effort to begin to identify specific protein fragments that are altered with radiation exposure. Computer-based analyses of the SELDI protein spectra could distinguish unexposed from radiation-exposed patient samples with 91–100 % sensitivity and 97–100 % specificity using various classifier models. The method also showed an ability to distinguish high from low dose-volume levels of exposure with a sensitivity of 83–100 % and specificity of 91–100 %. Using direct identity techniques of albumin-bound peptides, known to underpin the SELDI-TOF fingerprints, unique protein fragments/peptides were detected in the radiation exposure group, including an IL-6 precursor protein. The composition of proteins in serum seems to change with ionizing radiation exposure.

A combination of genome-wide association study (GWAS), gene expression, radiation response, and gene knockdown has narrowed in on five genes that seem to be associated with radiation response in both lymphoblastoid cell lines and specific cancer cell lines (Niu et al. 2010). Functional validation using siRNA knockdown in multiple tumor cell lines showed that C13orf34, MAD2L1, PLK4, TPD52, and DEPDC1B each significantly altered radiation sensitivity in at least two cancer cell lines. Such studies can help to identify novel biomarkers that might contribute to variation in response to radiation therapy and enhance our understanding of mechanisms underlying the variation in response to radiation. That might help to maximize radiation efficiency in the tumor while minimizing side effects in normal tissues. The work may ultimately lead to biomarkers for individualizing radiation therapy based on the expression of these candidate genes and may make it possible to design novel combination therapy for selected patients based on these biomarkers to overcome resistance.

5.4.11 Safety Biomarkers in Oncology Studies

In the conduct of clinical studies of anticancer agents administered to patients with advanced malignancy, safety biomarker results are playing an increasingly important role. Safety biomarkers may be appropriately used for decision-making by the application of uniform criteria, especially in situations where there is a degree of correlation between biomarker changes and corresponding clinical outcomes. While new safety biomarkers have major value, their applications require careful consideration to avoid unintended consequences that could negatively affect patient care and the development of promising new oncology therapeutics.

5.4.12 Role of Biomarkers in Phase I Clinical Trials of Anticancer Drugs

A new model has been suggested of early clinical trial design involving patient selection through predictive biomarkers for selected molecularly targeted agents (Carden et al. 2010). This model can maximize the chances of patient benefit and the yield of biological and clinical information as well as direct subsequent clinical trials. Ultimately, this may result in a new paradigm of drug development, focused on patients with tumors with the same oncogenic molecular abnormalities, rather than focused on patients with tumors from the same anatomical site or similar histopathology. Such biomarkers, predicting response to molecular-targeted agents, have the potential for selecting patients for these trials who are more likely to benefit from the treatment. This may facilitate early experience of and steps toward clinical qualification of predictive biomarkers, generate valuable information on cancer biology, and enable development of personalized anticancer therapy. New models of phase I study design of cancer that incorporate patient selection based on predictive biomarkers have the potential to accelerate anticancer drug development for many molecular-targeted novel agents. Indeed, it is probable that the early identification of such predictive biomarkers will improve the odds of eventual drug registration.

A genomic-based approach has been used to identify pharmacodynamic biomarkers for a cyclin-dependent kinase inhibitory drug, R547 (Roche), which is a potent cyclin-dependent kinase inhibitor with a potent antiproliferative effect at pharmacologically relevant doses and is currently in phase I clinical trials (Berkofsky-Fessler et al. 2009). Using preclinical data derived from microarray experiments, they identified pharmacodynamic biomarkers for further testing in blood samples from patients in clinical trials. The selection of candidate biomarkers was based on several criteria: relevance to the mechanism of action of R547, dose responsiveness in preclinical models, and measurable expression in blood samples. They identified 26 potential biomarkers of R547 action and tested their clinical validity in patient blood samples by quantitative real-time PCR analysis. Based on

the results, eight genes (FLJ44342, CD86, EGR1, MKI67, CCNB1, JUN, HEXIM1, and PFAAP5) were selected as dose-responsive pharmacodynamic biomarkers for phase II clinical trials.

5.5 Biomarkers According to Organ/Type of Cancer

There is no general biomarker for cancer. Since the tumors involving different organs differ considerably, biomarkers have been investigated according to the type of cancer.

5.5.1 Bladder Cancer Biomarkers

Tumors arising from the urothelial mucosa lining the urinary bladder are the most common malignancy of bladder and upper urinary tract (ureters and renal pelvis). Proteomic techniques have been used to systematically identify the proteins in urine samples of patients with squamous cell carcinoma of the bladder, identifying a protein called psoriacin as an early biomarker for the disease.

A multitarget, multicolor FISH assay has been developed for the detection of urothelial carcinoma in urine specimens. A FISH assay containing centromeric probes to chromosomes 3, 7, and 17 and a locus-specific probe to band 9p21 has high sensitivity and specificity for the detection of bladder cancer from voided urine specimens. UroCor Inc., using Ambion's technology, has developed a test for direct identification of p53 gene mutations in patients with bladder cancer utilizing a urine specimen.

5.5.1.1 Detection of FGFR3 Mutations in Urine for Diagnosis of Bladder Cancer

Fibroblast growth factor receptor 3 (FGFR3), an important and well-established DNA biomarker, is present in 30–50 % of patients with bladder cancer, but also correlates with a lower rate of recurrence. Predictive Biosciences is developing and plans to commercialize a noninvasive urine-based diagnostic test for bladder cancer. A small sample of urine is collected and is subjected to NGS to determine the presence of mutations. NGS enables higher sensitivity than qPCR. The exact percentage of mutated DNA in body fluids is unknown although an amount of 1 % has been detected. Predictive's ultradeep NGS-based assays for FGFR3 can detect a mutation when it is present in as little as 0.02 % of the total amount of DNA in urine. However, this is not possible at point-of-care, and the urine specimen has to be sent to a lab that specializes in NGS.

5.5.1.2 NMP22 BladderChek

NMP22 BladderChek (Matritech Inc.) is a point-of-care test for bladder cancer that returns results while the patient is in the doctor's office. Current tests performed in a central laboratory take 2–3 days to deliver results. NMP22 BladderChek measures the level of NMP22, a nuclear matrix protein. The test would be used in conjunction with cystoscopy, a procedure in which a fiber-optic tube is inserted into the bladder through the urethra permitting visual examination of the bladder.

5.5.1.3 Urinary Telomerase as Biomarker for Detection of Bladder Cancer

Telomerase is present in about 95 % of all epithelial cancers and therefore has great potential as a cancer biomarker. Expression levels of human telomerase reverse transcriptase (hTERT) and human telomerase RNA (hTR) can be analyzed by RT-PCR in urine samples from subjects with bladder cancer and controls with benign genitourinary diseases as well as healthy subjects. The sensitivity, specificity, and optimal cutoffs can be determined and compared to the corresponding values obtained by voided urine cytology. Quantitative urinary hTR analysis detects bladder cancer with an overall sensitivity of 77.0 %, whereas hTERT analysis reaches a sensitivity of 55.2 %. Both hTR and hTERT are significantly more sensitive than cytology. Higher diagnostic accuracy is achieved by hTR than by hTERT analysis. These data suggest that quantitative hTR analysis is the most accurate telomerase-based test for bladder cancer detection and has the potential to replace cytology as a noninvasive biomarker for disease diagnosis and follow-up. A noninvasive urine assay for bladder cancer, based on telomerase biosensor technology, is being developed by Sienna Cancer Diagnostics (Melbourne, Australia).

5.5.1.4 Concluding Remarks About Biomarkers of Urinary Cancer

In general, the best new biomarkers give higher sensitivity than urinary cytology, but specificity is usually lower. By using new biomarkers, the intervals between follow-up cystoscopies can be increased and the detection of relapse can be improved. But to date no noninvasive biomarker has proven to be sensitive and specific enough to replace cystoscopy, either for the diagnosis or for the follow-up of bladder cancer. However, new biomarker combinations and algorithms for risk assessment hold promise for the future (Lintula and Hotakainen 2010).

5.5.2 Brain Tumor Biomarkers

The most common primary malignant tumor of the brain in adults is GBM. Routine diagnosis is based on brain imaging and histological examination. In the past, most

Table 5.4 Biomarkers of brain tumors

Cytogenetic biomarkers
EGFR gene amplification and BRAF rearrangement detected by FISH (fluorescence in situ hybridization)
Loss of heterozygosity (LOH) on chromosomes 1p, 19q, 17p, and 10q
Methylation profiling of brain tumors
Detection of methylation-dependent DNA sequence variation: methylSNP
Methylation of TMS1, an intracellular signaling molecule
MCJ as a biomarker of medulloblastoma
Protein biomarkers
Circulating microvesicles (exosomes) containing mRNA, miRNA, and angiogenic proteins
CSF protein profiling: N-myc oncoprotein, caldesmon, attractin
Receptor protein tyrosine phosphatase
Serum protein fingerprinting
Biomarkers of angiogenesis in brain tumors
VEGF-R2 levels in tumor tissues: in vivo evaluation of angiogenesis using molecular MRI
Metabolite biomarkers detected by magnetic resonance spectroscopy
N-acetylaspartate (diminished)
Choline
Lactate
miRNA
Biomarkers of response to therapy
Biomarkers to predict response to EGFR inhibitors
MRI biomarker for response of brain tumor to therapy
Biomarkers of prognosis of glioblastoma multiforme (GBM)
14-3-3zeta positive expression

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of the genetic studies of tumors involved cytogenetic analysis. Biomarkers are now used for various applications in brain tumors, e.g., to assess malignancy and guide therapy. In particular, the use of LOH and MSP is used clinically in several centers. Molecular techniques, such as LOH testing, FISH, DNA sequencing, and MGMT methylation status, are currently being employed in assessment of gliomas in some laboratories. Table 5.4 shows biomarkers of brain tumors.

5.5.2.1 14-3-3zeta Positive Expression as a Prognostic Biomarker for GBM

A clinical study has shown that 14-3-3zeta positive expression was observed in approximately 74.5 % of patients with GBM who had lower overall survival rates and median survival time than those in the 14-3-3zeta negative group (Yang et al. 2011). 14-3-3zeta positive expression in tumor cells also was correlated with a shorter interval to tumor recurrence. Univariate and multivariate analyses showed that 14-3-3zeta positive expression was an independent prognostic factor for GBM and can be used as a biomarker.

5.5.2.2 Biomarkers to Predict Response to EGFR Inhibitors

EGFRs are amplified and overexpressed in many different human cancers, a phenomenon generally associated with poor prognosis. Inhibitors of the tyrosine kinase activity associated with this receptor have been approved for the treatment of chemotherapy-refractory NSCLC and are in clinical trials for additional tumor types. While these inhibitors, gefitinib and erlotinib, display limited response rates when assessed in cohorts that include all patients, there are subgroups, defined by patient and tumor characteristics, that preferentially respond to these agents. An analysis of tumors obtained from a phase I trial of erlotinib in patients with GBM showed that patients whose tumors exhibited overexpression and amplification of EGFR responded better than patients who had normal levels of this gene and protein (Haas-Kogan et al. 2005). The phosphorylation state of PKB/Akt was also an important determinant for response, with low phospho-PKB/Akt levels predicting good response to erlotinib. These data underscore the importance of placebo-controlled trials to distinguish between prognostic indicators of disease progression more generally and predictive markers of response to therapy. Ultimately the goal of these studies is to allow selection of patients who will preferentially respond to EGFR inhibitors.

5.5.2.3 CD133 as Biomarker of Resistance to Radiotherapy

CSCs contribute to resistance of GBM to radiotherapy through preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity (Bao et al. 2006). The fraction of tumor cells expressing CD133 (Prominin-1), a biomarker for both neural stem cells and brain CSCs, rises after radiation in GBM. The radioresistance of CD133-positive glioma stem cells can be reversed with a specific inhibitor of the Chk1 and Chk2 checkpoint kinases. These results suggest that CD133-positive tumor cells represent the cellular population that confers GBM radioresistance and could be the source of tumor recurrence after radiation. Targeting DNA damage checkpoint response in CSCs may overcome this radioresistance and provide a therapeutic model for GBM.

5.5.2.4 Circulating Microvesicles as Biomarkers

GBM tumor cells release microvesicles (exosomes) containing mRNA, miRNA, and angiogenic proteins, which are taken up by normal host cells, such as brain microvascular endothelial cells. By incorporating an mRNA for a reporter protein into these microvesicles, it was demonstrated that messages delivered by microvesicles are translated by recipient cells (Skog et al. 2008). These microvesicles are also enriched in angiogenic proteins and stimulate tubule formation by endothelial cells. Tumor-derived microvesicles therefore serve as a means of delivering genetic information and proteins to recipient cells in the tumor environment. GBM microvesicles also stimulated proliferation of a human glioma cell line, indicating

a self-promoting aspect. mRNA mutant/variants and miRNAs characteristic of gliomas could be detected in serum microvesicles of GBM patients. The tumor-specific EGFRvIII was detected in serum microvesicles from several glioblastoma patients and can be considered a biomarker. Thus, tumor-derived microvesicles represent a new way of obtaining information about a cancer without a biopsy, offering a means of choosing the best therapy, seeing how a patient responds to treatment, and possibly offering a way to deliver therapies to the tumor. Exosome Diagnostics Inc. has licensed the technology for further development.

5.5.2.5 CSF Protein Profiling

Tumor-related proteins, N-myc oncoprotein and low molecular weight caldesmon (l-CaD), have been identified in CSF samples of patients with primary brain tumors. In one study, samples of CSF from patients with GBM, benign brain tumors, and mild TBI were collected and proteins bound to H4 chip were detected by SELDI-TOF MS, profiled, and then analyzed with artificial neural network algorithm (Liu et al. 2005a). The diagnostic model of CSF protein profiles for differentiating GBM from benign brain tumors was established and was challenged with the test set randomly, the sensitivity and specificity were 100 % and 91.7 %, respectively. Thus, SELDI-TOF MS, along with bioinformatic analysis tools, is an effective method for screening and identifying biomarkers of GBM.

5.5.2.6 CSF Attractin as a Biomarker of Malignant Astrocytoma

While using proteomic techniques to identify secreted proteins in the CSF samples of patients with various CNS diseases, attractin was consistently found to be elevated in the samples of patients with malignant astrocytoma (Khwaja et al. 2006). IHC confirmed that attractin is produced and secreted by the tumor cells. Furthermore, it was shown that CSF from brain tumor patients induces glioma cell migration and that attractin is largely responsible for this promigratory activity. Attractin is normally absent in the brain tissue. This study suggests that attractin is not only an important biomarker of malignant astrocytoma, but may also be an important mediator of tumor invasiveness and thus a potential target for future therapies.

5.5.2.7 ELTD1 as a Biomarker of Gliomas

Advanced data mining and a novel bioinformatic method was used to predict ELTD1 (EGF, latrophilin, and seven transmembrane domain-containing one on chromosome 1) as a potential novel biomarker that is associated with gliomas (Towner et al. 2013). Validation was done with IHC to detect levels of ELTD1 in human high-grade gliomas and rat F98 glioma tumors. In vivo levels of ELTD1 in rat F98

gliomas were assessed using molecular MRI. ELTD1 was found to be significantly higher in high-grade gliomas compared to low-grade gliomas (21 patients) and compared well to traditional IHC markers including VEGF, GLUT-1, CAIX, and HIF-1α. ELTD1 gene expression indicates an association with grade, survival across grade, and an increase in the mesenchymal subtype. This study strongly suggests that associative analysis was able to accurately identify ELTD1 as a putative glioma-associated biomarker. The detection of ELTD1 was also validated in both rodent and human gliomas, and may serve as an additional biomarker for gliomas in pre-clinical and clinical diagnosis of gliomas.

5.5.2.8 Methylation Profiling of Brain Tumors

Tumorigenesis is characterized by alterations of methylation profiles including loss and gain of 5-methylcytosine. In each GBM, hundreds of genes are subject to DNA hypermethylation at their CpG island promoters. A subset of GBMs is also characterized by locus-specific and genome-wide decrease in DNA methylation. Other epigenetic alterations, such as changes in the position of histone variants and changes in histone modifications are also likely important in the molecular pathology of GBM. Alterations in histone modifications are important as HDACs are targets for drugs in clinical trials for GBMs (Nagarajan and Costello 2009).

Methylation-dependent DNA sequence variation may be considered a sort of SNP (methylSNP). MethylSNPs can be easily converted into common SNPs of the C/T type by sodium bisulfite treatment of the DNA and afterward subjected to conventional SNP typing. SnaPshot™ and Pyrosequencing™ are adapted to determine the methylation of the test CpG in a quantitative manner. The adapted methods, called SNaPmeth and PyroMeth, respectively, give nearly identical results, but data obtained with PyroMeth shows less scattering. Furthermore, the integrated software for allele frequency determination from Pyrosequencing can be used directly for data analysis, while SNaPmeth data has to be exported and processed manually.

TMS1/ASC is an intracellular signaling molecule with proposed roles in the regulation of apoptosis. Whereas normal brain tissue is unmethylated at the TMS1 locus and expresses TMS1 message, human GBM cell lines exhibit reduced or absent expression of TMS1 that is associated with aberrant methylation of a CpG island in the promoter of the TMS1 gene. Progression of GBM from grade III to grade IV is associated with selective expansion of TMS1-negative cells. These findings suggest a role for the epigenetic silencing of TMS1 in the pathogenesis of human GBM. Methylation of TMS1 may prove to be a useful prognostic biomarker and/or predictor of patient survival and tumor malignancy.

Patients with GBM have large amounts of DNA in the plasma, and methylated promoters that are frequently present in the tumor are also found in the plasma. This represents the first step to development of a quantitative plasma biomarker that could be used to monitor glioma status. Certain molecular biomarkers, in particular MGMT promoter hypermethylation, are associated with response to alkylating chemotherapy and longer survival in GBM patients.

MCJ (DNAJD1) is a member of the DNAJ protein family whose expression is controlled epigenetically by methylation of a CpG island located within the 5′ transcribed region of its gene. Extensive methylation patterns are associated with the methylation-dependent transcriptional silencing of MCJ in medulloblastoma and further investigations of the mechanism of MCJ inactivation have revealed that its loss could occur either through biallelic epigenetic methylation or by methylation in association with genetic loss of its second allele. These data indicate that epigenetic inactivation of MCJ may play a role in the development of a range of pediatric brain tumors and its role in pathogenesis and chemotherapeutic resistance should now be investigated further.

Certain molecular biomarkers, in particular MGMT promoter hypermethylation, are associated with response to alkylating chemotherapy and longer survival in GBM. Specimens from patients who were treated by open resection of the tumor, followed by radiotherapy and adjuvant temozolomide chemotherapy, were investigated for MGMT promoter methylation, mRNA, and protein expression, as well as presence of MGMT sequence polymorphisms (Felsberg et al. 2009). In addition, they were screened for genetic aberrations of the EGFR, TP53, CDK4, MDM2, and PDGFRA genes as well as allelic losses on chromosomal arms 1p, 10q, and 19q. Correlation of molecular findings with clinical data revealed significantly longer time to progression after onset of chemotherapy and longer overall survival of patients with MGMT-hypermethylated tumors. In contrast, MGMT protein expression, MGMT polymorphisms, and aberrations in any of the other genes and chromosomes were not significantly linked to patient outcome. Multivariate analysis identified MGMT promoter hypermethylation and near-complete tumor resection as the most important parameters associated with better prognosis. This study provides novel insights into the significance of molecular and clinical biomarkers in predicting the prognosis of glioblastoma patients, which may improve stratification of patients into distinct prognostic subgroups.

5.5.2.9 Metabolite Biomarkers of Brain Tumors

Metabolite biomarkers of brain tumors are detected by MRS. This technique used to study a few metabolites in the brain or tumors in situ and can provide information on tumor histological type and grade as well as for monitoring treatment.

In the normal brain, the largest signals arise from *N*-acetylaspartate (NAA), which is confined to neurons but absent in glial cells. NAA signal is diminished or absent in brain tumors. Choline is a neurotransmitter and a component of the cell membranes. An increase in choline signal is also characteristic of brain tumors. It may indicate rapid cell division or a necrotic process associated with tumor. Lactate is an end product of anaerobic metabolism that occurs when a rapidly growing tumor does not get enough oxygen from its neovasculature. Its presence indicates cellular breakdown. Some brain tumors may show a lactate signal that is normally inverted.

3D MRS imaging is applied and continuously improved at the Magnetic Resonance Science Center of the University of California (San Francisco, CA). The current protocol uses a 3D chemical shift imaging and multivoxel MRS imaging at the conclusion of a diagnostic MRI study. The raw data are reconstructed using special software. Initial studies at this center have focused on characterizing the spectra based on relative values of choline and NAA.

5.5.2.10 miRNAs as Biomarkers of Brain Tumors

Impairment of miRNA regulatory network is one of the key mechanisms in pathogenesis of GBM. miRNA deregulation is involved in cell proliferation, apoptosis, cell cycle regulation, invasion, glioma stem cell behavior, and angiogenesis (Novakova et al. 2009). The analysis of both GBM tissues and GBM cell lines has enabled identification of a group of miRNAs whose expression is significantly altered in GBM. miR-221 is strongly upregulated in GBM, whereas miR-128, miR-181a, miR-181b, and miR-181c, from a set of brain-enriched miRNAs, are downregulated.

miR-15a and miR-16-1 genes are located at chromosome 13q14, a region which is frequently deleted in pituitary tumors. miR-15a and miR-16-1 are expressed at lower levels in pituitary adenomas as compared to normal pituitary tissue. Downregulation of miR15 and miR16 in pituitary adenomas correlates with a greater tumor diameter and a lower p43 secretion, suggesting that these genes may, at least in part, influence tumor growth (Bottoni et al. 2005).

5.5.2.11 MRI Biomarker for Response of Brain Tumor to Therapy

Assessment of radiation and chemotherapy efficacy for brain cancer patients is traditionally accomplished by measuring changes in tumor size several months after therapy has been administered. The ability to use noninvasive imaging during the early stages of fractionated therapy to determine whether a particular treatment will be effective would provide an opportunity to optimize individual patient management and avoid unnecessary systemic toxicity, expense, and treatment delays. In a clinical study, brain tumor patients were examined by standard and diffusion MRI before initiation of treatment (Moffat et al. 2005). Additional images were acquired 3 weeks after initiation of chemo- and/or radiotherapy. Images were coregistered to pretreatment scans, and changes in tumor-water diffusion values were calculated and displayed as a functional diffusion map (fDM) for correlation with clinical response. The fDMs were found to predict patient response at 3 weeks from the start of treatment, revealing that early changes in tumor diffusion values could be used as a prognostic indicator of subsequent volumetric tumor response. Overall, fDM analysis provides an early biomarker for predicting treatment response in brain tumor patients.

5.5.2.12 Multigene Predictor of Outcome in GBM

No single biomarker is a predictor of outcome in GBM. An analysis using GBM microarray data from four independent datasets of the genes consistently associated with patient outcome revealed a consensus 38-gene survival set (Colman et al. 2010). Worse outcome was associated with increased expression of genes associated with mesenchymal differentiation and angiogenesis. Application to formalin-fixed, paraffin embedded (FFPE) samples using real-time RT-PCR assays resulted in a 9-gene subset which appeared robust in these samples. This 9-gene set was then validated in an additional independent sample set. Multivariate analysis confirmed that the 9-gene set was an independent predictor of outcome after adjusting for clinical factors and methylation of the methylguanine methyltransferase promoter. The 9-gene profile was also positively associated with biomarkers of glioma stemlike cells, including CD133 and nestin. Finally, a multigene predictor of outcome in GBM was identified, which is applicable to routinely processed FFPE samples. The profile has potential clinical application both for optimization of therapy in GBM and for the identification of novel therapies targeting tumors refractory to standard therapy. The assay is commercially available as DecisionDx-GBM (Castle Biosciences Inc.).

5.5.2.13 Neuroimaging Biomarkers Combined with DNA Microarray Analysis

Combined neuroimaging and DNA microarray analysis have been used to create a multidimensional map of gene expression patterns in GBM that provides clinically relevant insights into tumor biology (Diehn et al. 2008). Tumor contrast enhancement and mass effect can predict activation of specific hypoxia and proliferation gene expression programs, respectively. Overexpression of EGFR, a receptor tyrosine kinase and potential therapeutic target, has also been directly inferred by neuroimaging and validated in an independent set of tumors by IHC. Furthermore, imaging provides insights into the intratumoral distribution of gene expression patterns within GBM. An "infiltrative" imaging phenotype can identify and predict patient outcome. Patients with this imaging phenotype have a greater tendency toward having multiple tumor foci and demonstrate significantly shorter survival than their counterparts. This study demonstrates a simple, widely applicable method for discovering imaging biomarkers that are associated with underlying gene expression signatures. This approach facilitates the association of complex molecular signatures with readily identifiable imaging characteristics. These findings provide an in vivo portrait of genome-wide gene expression in GBM and offer a potential strategy for noninvasively selecting patients who may be candidates for individualized therapies.

5.5.2.14 Receptor Protein Tyrosine Phosphatase β as Biomarker of Gliomas

The receptor protein tyrosine phosphatase β (RPTPβ) is a functional biomarker for several solid tumor types. RPTPβ expression is largely restricted to the CNS and overexpressed primarily in astrocytic tumors. RPTPβ is expressed in a variety of solid tumor types with low expression in normal tissue. RPTPβ is known to facilitate tumor cell adhesion and migration through interactions with extracellular matrix components and the growth factor pleiotrophin. It is possible to generate MAbs that selectively recognize RPTPβ and kill glioma cells.

5.5.2.15 Serum Protein Fingerprinting

Screening and evaluation of protein biomarkers for the detection of GBM and their distinction from healthy individuals and benign gliomas has been done by using surface-enhanced laser desorption/ionization–time-of-flight mass spectrometry (SELDI-TOF MS) coupled with an artificial neural network algorithm (Liu et al. 2005b). An accuracy of 95.7 %, sensitivity of 88.9 %, specificity of 100 %, positive predictive value of 90 %, and negative predictive value of 100 % were obtained in a blinded test set comparing gliomas patients with healthy individuals; an accuracy of 86.4 %, sensitivity of 88.9 %, specificity of 84.6 %, positive predictive value of 90 %, and negative predictive value of 85.7 % were obtained when patient's gliomas was compared with benign brain tumor. Total accuracy of 85.7 %, with accuracy of grade I–II astrocytoma of 86.7 %, and accuracy of III–IV astrocytoma of 84.6 % were obtained when grade I–II astrocytoma was compared with grade III–IV ones (discriminant analysis). SELDI-TOF MS combined with bioinformatic tools could greatly facilitate the discovery of better biomarkers. The high sensitivity and specificity achieved by the use of selected biomarkers showed great potential application for the discrimination of gliomas patients from healthy individuals and gliomas from brain benign tumors.

5.5.2.16 VEGF-R2 as Biomarker of Angiogenesis in Brain Tumors

The level of VEGF-R2 (vascular endothelial growth factor receptor 2) is elevated during angiogenesis, and its levels in tumor tissues can be measured in Western blots and via IHC. A MRI molecular probe has been developed for the in vivo detection of VEGF-R2 in an experimental rodent model of disease glioma (Towner et al. 2010). The molecular-targeting agent that was used in this study incorporated a magnetite-based dextran-coated NP backbone covalently bound to an anti-VEGF-R2 antibody. Molecular MRI with an anti-VEGF-R2 probe was shown to detect in vivo VEGF-R2 levels as a molecular biomarker for gliomas (primary brain tumors). Prussian blue staining for iron-based nanoprobes was used to confirm the specificity of the probe for VEGF-R2 in glioma tissue. Another application of this technique is in vivo assessment of angiogenesis for tissue engineering.

5.5.3 Bone Tumor Biomarkers

Bone tumors are rare but have wide spectrum from benign to malignant. Bone tumors along with soft tissue cancers make up about 1 % of all new cancer cases, but 10 % of cancers in children and adolescents. Pathophysiology of bone tumors is still not well understood, and there are technical problems of processing bone specimens for molecular studies.

5.5.3.1 Cytogenetics for the Study of Bone and Soft Tissue Tumors

Classical as well as molecular cytogenetics has been used to examine tumors for the presence of DNA abnormalities. Molecular genetic analysis of chondrosarcomas has revealed some of the abnormalities responsible for the traits of the malignant phenotype. Specific chromosomal aberrations have been described in sarcomas, which may be divided into two subgroups based on cytogenetics, one with near-diploid karyotype and few chromosomal changes, but with specific translocations, and one with complex karyotypes and multiple cytogenetic aberrations. Sarcomas should be karyotyped in order to identify chromosomal changes in general, whereas FISH and PCR are the most common methods for detecting the relevant, specific translocations. Cytogenetics has been used for differentiating between benign and malignant soft tissue as well as bone tumors. For example, lipoma has 12q13, 6p, and 13q changes, but liposarcoma (myxoid and round cell) shows t(12;16)(q13;p11) rearrangement.

5.5.3.2 Biomarkers of Ewing's Tumors

Ewing's sarcoma family tumors (ESFTs), which affect both bone and soft tissues, include Ewing's sarcoma (EWS) and primitive neuroectodermal tumor. ESFTs are aggressive tumors of putative stem cell origin for which prognostic biomarkers and novel treatments are needed. Histologically, these tumors are composed of sheets of small round cells with minimal cytoplasm. Immunohistochemical biomarkers greatly facilitate differentiating these lesions from mimics. In particular, CD99 overexpression can be detected in most tumors. Early cytogenetic analysis points to a characteristic t(11;22) translocation. Molecular methods used to facilitate the diagnosis of EWS generally include FISH and RT-PCR. FISH for EWS using the break-apart technique has the advantage of detecting a broad array of translocations, making this a useful screening test; a small number of translocation negative cases may be resolved by also assessing for the translocation of fusion genes. Proteomic studies have shown that nucleophosmin is a prognostic biomarker of EWS that correlates with survival (Kikuta et al. 2009).

BMI-1 Polycomb protein is overexpressed by the vast majority of ESFTs and is associated with poor prognosis. However, in 20 % of cases, BMI-1 levels are low or undetectable. Although clinical presentation and outcome are similar between

BMI-1-high and BMI-1-low tumors, whole-genome expression array analysis shows marked differences in their respective gene expression profiles. Gene-specific enrichment analysis has identified several cancer-associated canonical biological pathways, including IGF1, mammalian target of rapamycin (mTOR), and WNT, which are significantly downregulated in BMI-1-low compared to BMI-1-high tumors (Cooper et al. 2011). Consistent with these in vivo data, the response to IGF1-R inhibition in vitro is diminished in BMI-1-low compared with BMI-1-high ESFT cells.

5.5.3.3 Role of Biomarkers in the Diagnosis of Bone Tumors

If molecular biomarkers are used in the diagnosis of bone tumors (e.g., EWS), they should not form the basis of the diagnosis by themselves; this information should be integrated with the clinical, radiological, and pathological features of the tumor. Ultrastructural, immunohistochemical, cytogenetic, and molecular studies should be used to supplement histological observations. Whole-genome sequencing from formalin-fixed, paraffin-embedded tissue promises to provide a wealth of information regarding bone tumor genetics (Dickson and Kandel 2010).

5.5.4 Breast Cancer Biomarkers

Breast cancer is one of the most common diseases affecting women. More than 250,000 women are diagnosed with breast cancer every year in the USA. According to the National Cancer Institute, approximately 13 % of women in the USA will develop breast cancer during their lifetime. The cause of breast cancer is multifactorial, involving environmental, hormonal, and genetic factors. Early detection of breast cancer is important for management and prognosis. Mammography and ultrasound are the most commonly used among the various methods used currently for screening for breast cancer. Various biomarkers for cancer are shown in Table 5.5. Important biomarkers of breast cancer include genes and HER-2/neu oncoprotein.

5.5.4.1 Autoantibody Biomarkers of Breast Cancer

Biomarkers are potentially useful for diagnosis of basal breast tumors, which are particularly difficult to diagnose with mammograms. Similar to these cancers, estrogen receptor (ER)-negative breast cancers are detected through other methods as they often occur in younger women, who tend to have denser breast tissue, which makes mammography less successful. These women could also benefit from an additional blood test to pick up biomarkers. Autoantibodies are promising

Table 5.5 Biomarkers of breast cancer

Biomarkers of breast cancer
Detection of predisposition to breast cancer
Cancer gene profiling: BRCA1, BRCA2, and EMSY
Proteomic biomarkers for early detection of breast cancer
Autoantibody biomarkers of breast cancer
Cdk6
Epithelial growth factor receptor (EGFR) levels in blood elevated due to mutations of EGFR gene
High mobility group protein A2
Mammaglobin
Progranulin
Riboflavin carrier protein
Serum proteomic profiling
Suppressor of deltex protein
Breath biomarkers of breast cancer: mixture of organic volatile compounds
Antigens as biomarkers of breast cancer
Serum CEA: prognostic factors for locally advanced breast cancer
Proliferating cell nuclear antigen: isoform associated with malignancy
miRNA biomarkers of breast cancer
Biomarkers for identifying patients at high risk for distant metastases
Cyclin E
CEACAM6 (carcinoembryonic antigen-related cell adhesion molecule 6)
Dachshund gene (DACH1)
Gene ratio: homeobox 13 (HOXB13) and interleukin-17B receptor (IL17BR)
Hypermethylation biomarkers: 14-3-3-δ gene
Podocalyxin: a CD34-related transmembrane protein
Protein kinase C epsilon
UPA/PAI-1
Predictive biomarkers for response to therapeutic agents
Prediction of response to endocrine therapy: estrogen receptor/progesterone receptor
Predictors of response to antiestrogen therapy: retinoblastoma tumor suppressor gene
Predictors of response to cytotoxic chemotherapy
Predictors of response to chorionic gonadotropin
Predictors of response to tamoxifen (decrease of level of insulin-like growth factor)
Predictors of response to trastuzumab (Herceptin): HER 2 gene overexpression
Biomarkers of prognosis of breast cancer
Carbonic anhydrase IX (CAIX) indicates poor prognosis in postmenopausal women with breast cancer
Centromere protein-F (CENP-F) is a gene associated with poor prognosis in breast cancer
Cytokeratins
GP88: elevation in women with ER+ breast cancer is associated with fourfold increased risk of progression
High expressing levels of p16 and/or COX-2, when coupled with tumor proliferation (low or high)
Lipocalin 2 (Lcn2) promotes breast cancer progression
p27 expression as biomarker for survival after treatment with adjuvant chemotherapy
Serum CA 15-3 and CA 27-29: prognostic factors for locally advanced breast cancer
Type III TGF-β receptor as regulator of cancer progression

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blood-based markers. Although individual autoantibodies are unlikely to enhance early detection, multiplexed assays for autoantibody panels may achieve the required sensitivity. A technology under development at Arizona State University, NAPPA (Nucleic Acid Programmable Protein Arrays), enables creation of high-density, customized protein arrays that could be used for this purpose. The idea is to display several different proteins in a sample so the autoantibodies in a patient's serum can find any proteins they happen to recognize. The arrays need to be fairly stable and should display proteins that will not change or unfold over time. The NAPPA approach is to take cloned copies of genes and print them on the array. The cloned copies, cDNA, and are configured in such a way that an epitope tag can be added at the C-terminus of the gene. Anything that is captured by virtue of the tag must have the full-length protein attached to it. Those genes are printed on the array, which can then be stored for months. Once the array is needed, it is floated in expression extract that transcribes and translates the proteins in situ on the glass. Those proteins are made about an hour before they are used, guaranteeing that they are as fresh as possible.

The technology uses minimal samples, as little as 0.01 mL of plasma or serum, and has good assay reproducibility and reliability based on pilot work, making it particularly attractive for molecular epidemiology studies. NAPPA technology and other proteomic platforms are another extension of genomic technologies that might provide novel biomarkers for breast cancer. However, this is a new area and the biology of autoantibodies is not well understood. There is need to learn how age and lifestyle factors, as well as intra-person and inter-person variability, influence these biomarkers. Because basal breast cancers are highly aggressive, it may not be possible to isolate autoantibody biomarkers appropriate for early detection, and one may only find biomarkers related to overall tumor aggressiveness. The ultimate goal of the research is to identify candidate autoantibody biomarkers that may detect basal breast cancers early or predict survival.

5.5.4.2 Biomarkers of Breast Cancer in Breath

A breath test for volatile organic compounds (VOCs) as a predictor of breast cancer has been developed by Menssana Research Inc. Breath VOCs were assayed in asymptomatic women with abnormal mammograms and biopsy-proven breast cancer and compared to control subjects of age-matched healthy women. A fuzzy logic model predicted breast cancer with accuracy superior to previously reported findings. Following random assignment to a training set or a prediction set, a model was constructed in the training set employing five breath VOCs that predicted breast cancer in the prediction set with 93.8 % sensitivity and 84.6 % specificity. The same model predicted no breast cancer in 32.0 % of women with abnormal mammograms and no cancer on biopsy. A 2-min breath test could potentially provide a safe, accurate, and painless screening test for breast cancer, but prospective validation studies are required. Such studies are being conducted.

5.5.4.3 Biomarkers for Breast Cancer in Nipple Aspiration Fluid

Breast fluid is a rich source of breast cancer biomarkers. Ductal lavage is a method of minimal epithelial sampling of the breast, with potential utility for repeat sampling and biomarker analysis. In combination with high-throughput novel proteomic profiling technology and multicenter study design, biomarkers that are highly specific to breast cancer can be discovered and validated.

Persistent elevation of human neutrophil peptide in high-risk women may imply early onset of cancer not yet detectable by current detection method. Proof of this hypothesis requires follow-up on a larger study population.

5.5.4.4 Circulating Tumor DNA as Biomarker of Breast Cancer

Monitoring of response to treatment of breast cancer is essential for avoiding continual of ineffective therapies to prevent unnecessary side effects, and to determine the benefit of new therapies. Treatment response is generally assessed with the use of serial imaging, but radiographic measurements often fail to detect changes in tumor burden. Serum biomarker CA 15-3 is clinically useful in some patients with MBC, but it has a sensitivity of only 60–70 %. Therefore, there is an urgent need for biomarkers that measure tumor burden with high sensitivity and specificity.

Circulating nucleic acids (CNAs) isolated from serum or plasma are increasingly recognized as biomarkers for cancers. NGS provides high numbers of DNA sequences to detect the trace amounts of unique serum biomarkers associated with breast carcinoma. Serum CNA of women with ductal carcinoma was extracted and sequenced on a 454/Roche high-throughput GS-FLX platform and compared with healthy controls and patients with other medical conditions (Beck et al. 2010). Breast cancer was accurately detected at a diagnostic specificity level of 95 % with a calculated sensitivity of 90 %. Identification of specific breast cancer-related CNA sequences provides the basis for the development of a serum-based routine laboratory test for breast cancer screening and monitoring. This is in development by Chronix Biomedical.

For the detection of MBC, circulating tumor DNA shows superior sensitivity to that of other circulating biomarkers and has a greater dynamic range that correlates with changes in tumor burden (Dawson et al. 2013). Circulating tumor DNA often provides the earliest measure of treatment response.

5.5.4.5 Flow Cytometry for Quantification of Biomarker Expression Patterns

The established method in prognosis of breast cancer includes detection of molecular markers, such as the ER, progesterone receptor (PR), and HER-2/neu. These markers are routinely checked via IHC. HER-2/neu is also detected by FISH. Flow cytometric analysis can provide quantitative data on expression patterns of

important prognostic markers in breast cancer and has been used for the detection of ER, PR, HER-2/neu, EGFR, and E-cadherin. Currently, EGFR and E-cadherin are not standard predictive factors in determining survival of breast cancer patients, but both may be beneficial for determining prognosis in the future. Cells undergoing flow cytometric analysis lose marker expression with increasing passage number. The highest expression is found at cells passaged 0–1 times. ER, PR, and HER-2/neu marker expressions in 5 out of 5 cell lines were consistent with established expression patterns. EGFR and E-cadherin expression in 4 out of 5 cell lines were also consistent with established expression patterns.

5.5.4.6 Plasma Proteomics for Biomarkers of Breast Cancer

Protein-based breast cancer biomarkers are a promising resource for breast cancer detection at the earliest and most treatable stages of the disease. Plasma is well suited to proteomic-based methods of biomarker discovery because it is easily obtained, is routinely used in the diagnosis of many diseases, and has a rich proteome. However, due to the vast dynamic range in protein concentration and the often uncertain tissue and cellular origin of plasma proteins, proteomic analysis of plasma requires special consideration compared with tissue and cultured cells. For example, when studies report an upregulation of IL-6 in the serum of breast cancer patients compared with control individuals, it is difficult to know whether this protein is released directly from the tumor or whether IL-6 upregulation is a systemic reaction to the tumor and released by nontumor tissues.

Biomarkers should be tissue specific in addition to being tumor specific. If cancer is detected, but not the tissue of origin, it may create problems, since searching for a suspected tumor will add undue stress to the patient and increased cost to the treatment. Finding tissue-specific tumor markers has thus far proven difficult. Many candidate biomarkers have been concurrently identified in numerous tumor types. This likely reflects the fact that 90 % of all cancers are of epithelial origin and thus express many of the same proteins. It is probable that a panel of biomarkers will be required to establish tissue specificity rather than a single protein; this panel may or may not be independent of a tumor-specific panel of biomarkers. In addition, early detection biomarkers may need to be used in conjunction with other screening methods, such as mammography, where the tissue of origin is not in question.

5.5.4.7 Real-Time Quantitative PCR Assays for Biomarker Validation

Microarray analysis and a real-time qPCR assay have been used to stratify risk in breast cancer based on biological “intrinsic” subtypes and proliferation. Real-time qPCR is attractive for clinical use because it is fast, reproducible, tissue-sparing, quantitative, automatable, and can be performed from archived FFPE tissue samples. The benefit of using real-time qPCR for cancer diagnostics is that new

biomarkers can be readily validated and implemented, making tests expandable and/or tailored to the individual. For instance, the proliferation metagene could be used within the context of the intrinsic subtypes or used as an ancillary test in breast cancer and other tumor types where an objective and quantitative measure of grade is important for risk stratification. As more prognostic and predictive signatures are discovered from microarray, it should be possible to build on the current biological classification and develop customized assays for each tumor subtype. This approach enables the important clinical distinction between ER-positive and ER-negative tumors and identifies additional subtypes that have prognostic value. The proliferation metagene offers an objective and quantitative measurement for grade and adds significant prognostic information to the biological subtypes. It is a robust predictor of survival across all breast cancer patients and is particularly important for prognosis in ER-positive breast cancers, which have a worse outcome than expected when proliferation is high. This supports previous findings that a genomic signature of proliferation is important for predicting relapse in breast cancer, especially in ER-positive patients.

Comparison of real-time qPCR results of various studies for the assessment of mRNA levels of ERα, PgR, and the members of the human EGFR family, HER1, HER2, HER3, and HER4, show good concordance for all the six biomarkers. The quantitative mRNA expression levels of ERα, PgR, and HER2 also strongly correlates with the respective quantitative protein expression levels prospectively detected by EIA. In addition, HER2 mRNA expression levels correlated well with gene amplification detected by FISH in the same biopsies. These findings indicate that both real-time qPCR methods are robust and sensitive tools for routine diagnostics and consistent with standard methods. The simultaneous assessment of several biomarkers is fast as well as labor effective and optimizes clinical decision-making process in breast cancer tissue and/or core biopsies.

5.5.4.8 Cdk6 as a Biomarker of Breast Cancer

Normal human mammary epithelial cells have a high amount of cyclin-dependent kinase-6 (cdk6) protein and activity, but all breast tumor-derived cell lines that have been analyzed show reduced levels, with several having little or no cdk6, and these can be restored to those characteristic of normal human mammary epithelial cells by DNA transfection. According to researchers at the National Jewish Medical and Research Center (Denver, CO), cdk6 may be useful as a cancer biomarker and as a target for cancer therapy in patients with breast cancer. Potential applications are as follows:

- Diagnostic assay for breast cancer and for determining the stage of malignancy
- Predictor of in vivo tumor cell growth
- Diagnostic assays for evaluating the efficacy of anticancer treatments
- Method to regulate tumor cell growth

5.5.4.9 Centromere Protein-F

Reanalysis of a high profile breast cancer DNA microarray dataset containing 96 breast tumor samples and use of a powerful statistical approach, between group analyses, led to the identification of centromere protein-F (CENP-F), a gene associated with poor prognosis (O'Brien et al. 2007). In a published follow-up breast cancer DNA microarray study, comprising 295 tumor samples, CENP-F upregulation was found to be significantly associated with worse overall survival and reduced metastasis-free survival. To validate and expand upon these findings, the authors used two independent breast cancer patient cohorts represented on tissue microarrays. CENP-F protein expression was evaluated by IHC in 91 primary breast cancer samples from cohort I and 289 samples from cohort II. CENP-F correlated with markers of aggressive tumor behavior including ER negativity and high tumor grade. In cohort I, CENP-F was significantly associated with markers of cervical intraepithelial neoplasia (CIN) including cyclin E, increased telomerase activity, c-myc amplification, and aneuploidy. In cohort II, CENP-F correlated with VEGFR2, phosphorylated Ets-2 and Ki67, and in multivariate analysis was an independent predictor of worse breast cancer-specific survival and overall survival. In conclusion, CENP-F is associated with poor outcome in breast cancer.

5.5.4.10 Carbonic Anhydrase IX

Hypoxia in breast cancer is associated with poor prognosis and downregulation of the ER. Carbonic anhydrase IX (CAIX) is a hypoxia-inducible gene that has been associated with poor outcome in many epithelial cancers. Previous studies of CAIX in breast cancer have been carried out on mixed cohorts of premenopausal and postmenopausal patients with locally advanced disease and varying treatment regimens. Using tissue microarrays, the potential prognostic and predictive role of CAIX was examined in premenopausal breast cancer patients (Brennan et al. 2006). The patients had previously participated in a randomized control trial comparing 2 years of tamoxifen to no systemic adjuvant treatment. CAIX expression correlated positively with tumor size, grade, HIF-1α, Ki-67, cyclin E, and cyclin A2 expression. CAIX expression correlated negatively with cyclin D1, ER, and PR. CAIX expression was associated with a reduced relapse-free survival, overall survival, and breast cancer-specific survival. Multivariate analysis revealed that CAIX was an independent prognostic marker in untreated patients with one to three positive lymph nodes. It was concluded that CAIX is biomarker of poor prognosis in premenopausal breast cancer patients and it is an independent predictor of survival in patients with one to three positive lymph nodes. As all these patients received locoregional radiation therapy, CAIX may be associated with resistance to radiotherapy.

5.5.4.11 COX-2 as a Biomarker of Breast Cancer

Cyclooxygenase (COX) enzymes produce prostaglandin compounds responsible for pain and inflammation, and nonsteroidal anti-inflammatory drugs (NSAIDs) are designed to reduce expression of COX enzymes, although some NSAID use has been associated with side effects (most notably possible kidney failure). COX-2 is a form of COX that is not usually found in normal tissues but which has been associated with several cancers, including ductal carcinoma in situ (DCIS) and invasive breast cancers.

Atypical hyperplasia in breast tissue, although benign, is associated with a high risk of breast cancer. A study has assessed the relationship between risk of breast cancer and COX-2 expression in archival specimens from women with atypical hyperplasia and a 15-year follow-up (Visscher et al. 2008). The risk for developing breast cancer increased with increasing COX-2 expression. Overexpression of COX-2 was statistically significantly associated with the type of atypia, with number of foci of atypia in the biopsy, and with older age at time of biopsy. Specifically, 20 years after a biopsy in which atypia was found, 31 % of women with high levels of COX-2 in their atypia sample had developed breast cancer versus 14 % of those with no COX-2 expression. For those with moderate levels of COX-2, 24 % had developed breast cancer. This study indicates that COX-2 may be a biomarker that further stratifies breast cancer risk among women with atypia and may be a relevant target for chemoprevention strategies, e.g., COX-2 inhibitors such as celecoxib or rofecoxib.

5.5.4.12 G88 as a Biomarker of Progression of ER+ Breast Cancer

Out of 250,000 cases of breast cancer per year in the USA, ~175,000 are ER positive and receive a treatment regimen that includes antiestrogen compounds such as tamoxifen. Increased level of a protein biomarker GP88 (A&G Pharmaceutical) in these tumors is an indicator of fourfold increased risk of disease progression. Further clinical studies indicate that GP88 levels are elevated in breast cancer patients as compared to normal controls. A&G is developing a MAb against GP88.

5.5.4.13 Glycomic Biomarkers of Breast Cancer

Since the glycosylation of proteins is known to change in tumor cells during the development of breast cancer, a glycomic approach has been investigated to find relevant biomarkers of breast cancer. These glycosylation changes are known to correlate with increasing tumor burden and poor prognosis. Current antibody-based immunochemical tests for cancer biomarkers of ovarian (CA-125), breast (CA 27-29 or CA 15-3), pancreatic, gastric, colonic, and ovarian carcinoma (CA 19-9) target highly glycosylated mucin proteins. However, these tests lack the specificity and sensitivity for use in early detection. The glycomic approach to find glycan biomarkers of breast cancer involves chemically cleaving glycans from glycosylated

proteins that are shed or secreted by breast cancer tumor cell lines (Kirmiz et al. 2007). The resulting free glycan species are analyzed by MALDI Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR MS). Further structural analysis of the glycans can be performed in FTMS through the use of tandem MS with infrared multiphoton dissociation. These methods were then used to analyze sera obtained from a mouse model of breast cancer, and a small number of serum samples obtained from human patients diagnosed with breast cancer, or patients with no known history of breast cancer. In addition to the glycosylation changes detected in mice as mouse mammary tumors developed, glycosylation profiles were found to be sufficiently different so as to distinguish patients with cancer from those without. Although the small number of patient samples analyzed so far is inadequate to make any legitimate claims at this time, these promising but very preliminary results suggest that glycan profiles may contain distinct glycan biomarkers that may correspond to glycan "signatures of cancer."

5.5.4.14 HER-2/Neu Oncoprotein

Overexpression of members of the human epidermal growth factor receptor (HER) family has been widely studied in breast cancer. HER-2/neu oncoprotein has been widely studied for many years and has been shown to play a pivotal role in the development and progression of breast cancer. HER2/neu has been shown to be an indicator of poor prognosis with patients exhibiting aggressive disease, decreased overall survival, and a higher probability of recurrence of disease. Elevated levels of HER2/neu are found not only in breast cancer but also in several other tumor types including prostate, lung, pancreatic, colon, and ovarian cancers. As evidenced by numerous published studies, elevated levels of HER2/neu (also referred to as overexpression) are found in about 30 % of women with breast cancer. Determination of a patient's HER2/neu status may be valuable in identifying whether that patient has a more aggressive disease and would, thus, derive substantial benefit from more intensive or alternative therapy regimens. Some studies suggest that in certain breast cancer patients, persistently rising HER2/neu values may be associated with aggressive cancer and poor response to therapy, while decreasing HER2/neu levels may be indicative of effective therapy. The randomized, controlled Mammary5 trial by the National Cancer Institute of Canada showed that amplification of HER2 in breast cancer cells is associated with better clinical responsiveness to anthracycline-containing chemotherapy regimen when compared with the regimen of cyclophosphamide, methotrexate, and fluorouracil (Pritchard et al. 2006).

Traditional HER2/neu testing is generally limited to tissue from primary breast cancer and does not provide information regarding the HER2/neu status in women with recurrent, MBC. The introduction of microtiter plate ELISA HER-2/neu testing (Bayer Diagnostics) using a serum sample now offers a less invasive diagnostic tool and provides a current assessment of a woman's HER2/neu status over the course of disease. IHC analysis of HER2/neu in breast carcinoma is a useful predictor of response to therapy with trastuzumab when strongly positive. Negative immunostaining is highly

concordant with a lack of gene amplification by FISH. Most weakly positive overexpressors are false-positives on testing with FISH. Thus, screening of breast carcinomas with IHC and confirmation of weakly positive IHC results by FISH is an effective evolving strategy for testing HER2/neu as a predictor of response to targeted therapy. The clinical utility of the serum test as a prognostic indicator has not yet been fully established but is under investigation. Findings from a study using Bayer Diagnostics' serum HER2/neu test showed that proven MBC patients whose serum HER2/neu levels decreased by less than 20 % experienced decreased benefit from trastuzumab-based therapy.

Potential mechanisms of trastuzumab resistance include altered receptor–antibody interaction, increased cell signaling from other HER receptors, increased Akt activity, reduced PTEN level, reduced p27kip1, and increased IGF-IR signaling (Nahta et al. 2006). There is an urgent need to identify biomarkers to guide anti-HER2 therapy in patients, who develop progressive MBC while receiving trastuzumab, and to identify combination therapies using novel anti-HER2 agents. In a pooled analysis of patients with MBC, individuals who did not achieve a significant decline in serum HER-2/neu levels had decreased benefit from trastuzumab-based therapy, and these patients should be considered for clinical trials evaluating additional HER-2/neu-targeted interventions (Ali et al. 2008).

Current methods for checking HER2 are problematic because of issues with intra- and interlaboratory reproducibility and preanalytic variables, such as fixation time. In addition, the commonly used HER2/chromosome 17 ratio presumes that chromosome 17 polysomy is present when the centromere is amplified, even though analysis of the rest of the chromosome is not included in the assay. In one study, 97 frozen samples of invasive lobular and invasive ductal carcinoma, with known ICH and FISH results for HER2, were analyzed by aCGH to a commercially available bacterial artificial chromosome whole-genome array containing 99 probes targeted to chromosome 17 and the HER2/TOP2 amplicon (Yeh et al. 2009). Results were 97 % concordant for HER2 status, meeting the College of American Pathologists/American Society of Clinical Oncology's validation requirements for HER2 testing. No case of complete polysomy 17 was detected even though multiple breast cancer cases showed polysomies of other chromosomes. Therefore, aCGH is an accurate and objective DNA-based alternative for clinical evaluation of HER2 gene copy number and that polysomy 17 is a rare event in breast cancer. It is commercially available as HerScan™ (Combimatrix Molecular Diagnostics).

5.5.4.15 High Mobility Group Protein A2

High mobility group protein A2 (HMGA2) is a transcription factors that is expressed during embryonic development, but not in normal adult tissues. The HMGI family consists of three proteins, HMGI, HMGI(Y), and HMGA2 (also known as HMGI-C). Experiments with knockout HMGA2$^{-/-}$ mice yielded a reduced body weight compared to wild-type HMGA2$^{+/+}$ mice, which indicates that HMGA2 plays a role in mammalian growth. Interest in HMGA2 has increased recently as it has been found

that HMGA2 is expressed in neoplastic tissues and that it apparently has a role in control of cell growth, differentiation, and tumorigenesis. HMGA2 is expressed in tumor tissue but not in normal tissue immediately adjacent to the tumor tissue. Studies in peripheral blood show that HMGA2 is not present in normal healthy donors but present in the blood of a subset of breast cancer patients. In general, the presence of HMGA2 in the peripheral blood of breast cancer patients has a correlation with poor survival and with a higher histological grade of the tumor. The HMGA2 ELISA (OXIS International) is designed for the measurement of HMGA2 in cell culture supernatants, cell extracts, and tissue extracts. It is for research use only and not yet approved for use in humans.

5.5.4.16 Hypermethylated Genes as Biomarkers of Metastatic Breast Cancer

Numerous hypermethylated genes have been reported in breast cancer, and the silencing of these genes plays an important role in carcinogenesis, tumor progression, and diagnosis. These hypermethylated promoters are very rarely found in normal breast. Aberrant hypermethylation may be useful as a biomarker, with implications for breast cancer etiology, diagnosis, and management.

A study found that serum levels of methylated gene promoter 14-3-3-δ (Stratifin) significantly differed between control and MBC groups and between disease-free and MBC groups (Zurita et al. 2010). The ratio of the 14-3-3-δ level before the first chemotherapy cycle to the level just before administration of the second chemotherapy cycle, defined as the biomarker response ratio (BRR), was calculated for the "continuous decline" and "rise-and-fall" groups. Subsequent ROC analysis showed a sensitivity of 75 % and a specificity of 66.7 % for discriminating between the groups for a cutoff level of BRR = 2.39. The relationship of 14-3-3-sigma with breast cancer metastasis and progression found in this study suggests a possible application of 14-3-3-sigma as a biomarker to screen for metastasis and for follow-up of patients treated for MBC by monitoring their disease status and treatment response.

5.5.4.17 Lipocalin 2 as Biomarker of Breast Cancer Progression

Lipocalin 2 (Lcn2) promotes breast cancer progression, and the mechanisms underlying this function have been identified (Yang et al. 2009a). Lcn2 levels are consistently associated with invasive breast cancer in human tissue and urine samples. Lcn2 is overexpressed in human breast cancer cells and upregulates mesenchymal markers, including vimentin and fibronectin, downregulates the epithelial marker E-cadherin, and significantly increase cell motility and invasiveness. These changes in marker expression and cell motility are hallmarks of an epithelial–mesenchymal transition (EMT). In contrast, Lcn2 silencing in aggressive breast cancer cells inhibits cell migration and the mesenchymal phenotype. Furthermore, reduced expression of ER alpha and increased expression of the key EMT transcription factor Slug

are observed with Lcn2 expression. Overexpression of ERalpha in Lcn2-expressing cells reverses EMT and reduces Slug expression, suggesting that ERalpha negatively regulates Lcn2-induced EMT. Finally, Lcn2-expressing breast tumors display a poorly differentiated phenotype and show increased local tumor invasion and lymph node metastasis. Taken together, these in vitro, in vivo, and human studies demonstrate that Lcn2 promotes breast cancer progression by inducing EMT through the ERalpha/Slug axis and may be a useful biomarker of breast cancer.

5.5.4.18 Long Intervening Noncoding RNAs

Long intervening noncoding RNAs (lincRNAs) in the HOX loci become systematically dysregulated during breast cancer progression. The lincRNA termed HOTAIR is increased in expression in primary breast tumors and metastases, and HOTAIR expression level in primary tumors is a powerful predictor of eventual metastasis and death (Gupta et al. 2010). Enforced expression of HOTAIR in epithelial cancer cells induced genome-wide re-targeting of Polycomb repressive complex 2 (PRC2) to an occupancy pattern more resembling embryonic fibroblasts, leading to altered histone H3 lysine 27 methylation, gene expression, and increased cancer invasiveness and metastasis in a manner dependent on PRC2. Conversely, loss of HOTAIR can inhibit cancer invasiveness, particularly in cells that possess excessive PRC2 activity. TaqMan noncoding RNA assays (Life Technologies) can accurately measure expression levels of this molecular biomarker in different breast cancer samples and have helped to uncover regulatory roles of noncoding RNAs in breast cancer.

5.5.4.19 Mammaglobin

Mammaglobin, a glycoprotein, is almost exclusively expressed in breast epithelial cells. It is frequently elevated in breast cancer. An ELISA can detect mammaglobin and is highly sensitive and specific for detection of mammaglobin protein in tissue culture fluids of breast cancer cells and sera of breast cancer patients. Mammaglobin, as measured by the ELISA, holds significant promise for breast cancer screening with the realistic potential to impact management of this disease. Mammaglobin is also being explored as a target for immune-based interventions. In vitro studies have demonstrated that T-cell-mediated immune responses can be induced against mammaglobin-derived peptides expressed by MHC molecules on tumor cells and antigen-presenting cells.

5.5.4.20 miRNA Biomarkers of Breast Cancer

Altered abundance of cell cycle regulation proteins and aberrant expression of miRNAs frequently coexist in human breast cancers. Altered miRNA expression in breast cancer cell lines is associated with altered cell cycle progression and cell proliferation. Recent studies have demonstrated a causal role for miRNA in

Table 5.6 miRNA associated with breast cancer

miRNA	Expression status in fresh frozen breast cancer tissue specimens
miR-7	Expression associated with tumor aggressiveness
miR-10b	Downregulated in tumors in patients with distant and regional relapse and local recurrence
miR-21	Overexpression correlated with advanced clinical stage, lymph node metastasis, and poor prognosis
miR-125a	Downregulated in tumors
miR-125b	Downregulated in tumors
miR-128	Expression associated with tumor aggressiveness
miR-145	Downregulated in tumors
miR-155	Upregulated in tumors
miR-205	Downregulated in tumors
miR-206	Downregulated in ER+ tumors
miR-210	Expression associated with tumor aggressiveness, early relapse, and poor outcome
miR-93	Highly expressed in high-grade tumors
miR-106b	Highly expressed in high-grade tumors
miR-25	Highly expressed in high-grade tumors
miR-335	Increased expression led to metastasis suppression
miR-126	Increased expression led to metastasis suppression
miR-373	Increased expression stimulated cell migration and invasion
miR-516-3p	Expression associated with tumor aggressiveness
miR-520c	Increased expression stimulated cell migration and invasion
miR-27b	Downregulated in tumors
miR-17-5p	Downregulated in tumors
miR-9-1	Downregulated in tumors

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governing breast tumor suppression or collaborative oncogenesis (Yu et al. 2010). Various miRNAs associated with breast cancer are shown in Table 5.6.

Global testing pathway analysis showed an association of hsa-miRNA-30c expression with HER and RAC1 signaling pathways (Rodríguez-González et al. 2011). The authors identified hsa-miRNA-30c as an independent predictor for clinical benefit of tamoxifen therapy in patients with advanced breast cancer. Assessment of tumor levels and connected pathways could be helpful to improve treatment strategies.

5.5.4.21 p27 Expression as Biomarker for Survival After Chemotherapy

Abnormal expression of the cell cycle regulatory proteins p27(Kip1) (p27) and cyclin E may be associated with breast cancer survival and relapse. These biomarkers have been studied in clinical trials on patients with breast cancer treated by a uniform drug regimen and have shown that treatment is not associated with variability in outcome. Tissue microarrays are used to evaluate the expression of p27

and cyclin E proteins by IHC in tumor tissue from patients with moderate-risk primary breast cancer who are enrolled in clinical trials and assigned to receive doxorubicin and cyclophosphamide administered concurrently. Cyclin E expression is not statistically significantly associated with overall survival, but low p27 expression is associated with poor prognosis, especially among patients with steroid receptor-positive tumors. Five-year survival is >90 % in women whose tumors have high p27 expression, as compared to a survival rate of <85 % in women whose tumors exhibit low p27 expression. There is no association between p27 expression and decreased survival among women with hormone receptor-negative tumors. Although these observations suggest that p27 may be a useful biomarker for predicting breast cancer mortality, more work needs to be done before its widespread use. Currently, it appears to be most useful for predicting outcome and tailoring treatment in women whose tumors are hormone receptor positive.

5.5.4.22 Podocalyxin

Podocalyxin is a CD34-related transmembrane protein involved in hematopoietic cell homing and breast cancer progression but the mechanisms involved are not clear. It has, however, been postulated that the adaptor proteins NHERF-1 and NHERF-2 could regulate apical targeting of podocalyxin by linking it to the actin cytoskeleton. However, a new study has found that full-length podocalyxin acts to recruit NHERF-1 to the apical domain (Nielsen et al. 2007). Podocalyxin was found to significantly expand the nonadhesive face of cells, allowing individual cells to brush aside adhesion molecules situated between tumor cells. The freed cells then move away from the original site to form new tumors at other sites. Also, the protein causes tumor cells to sprout microvilli, or hair-like projections, that may help propel cancer cells to metastasize to other sites. The discovery demonstrated that the protein not only predicted the spread of breast cancer cells, it likely helped to cause it. The data from this study suggest that this single molecule can modulate NHERF localization and, independently, act as a key orchestrator of apical cell morphology, thereby lending mechanistic insights into its multiple roles as a polarity regulator, tumor progression marker, and anti-adhesin. The mechanism is now believed to apply to difficult-to-treat invasive breast and ovarian cancers. Next steps involve advancing the research in animal models, designing antibodies to block the function of the protein and identifying new therapies to combat metastasizing cancer.

5.5.4.23 Progranulin as a Biomarker of Breast Cancer

Progranulin (GP88/PGRN), which is produced by breast cancer, helps the tumor grow and proliferate. In clinical studies conducted by A&G Pharmaceutical, GP88 has been shown to consistently identify more than 80 % of breast cancers, which is significantly higher than any currently available method, and has the potential to detect a number of other cancers from blood samples and tissue biopsies. A&G is

currently focused on a near-term opportunity to complete development of and commercialize two GP88-based breast cancer diagnostic test kits to improve early detection, diagnosis, and treatment of breast cancer.

5.5.4.24 Proliferating Cell Nuclear Antigen

Two isoforms of proliferating cell nuclear antigen (PCNA) have been observed in breast cancer cells. Commercially available antibodies to PCNA recognize both isoforms and, therefore, cannot differentiate between the PCNA isoforms in malignant and nonmalignant breast epithelial cells and tissues. A unique antibody has been developed that specifically detects a PCNA isoform (caPCNA) associated with breast cancer epithelial cells grown in culture and breast tumor tissues (Malkas et al. 2006). Immunostaining studies using this antibody suggest that the caPCNA isoform may be useful as a biomarker of breast cancer and that the caPCNA-specific antibody could potentially serve as a highly effective detector of malignancy. It was also shown that the caPCNA isoform functions in breast cancer-cell DNA replication and interacts with DNA polymerase delta. These studies indicate that the caPCNA isoform may be a previously uncharacterized detector of breast cancer.

5.5.4.25 Protein Kinase C as a Predictive Biomarker of Metastatic Breast Cancer

Protein kinase C epsilon (PKC epsilon), a member of a family of serine/threonine protein kinases, is a transforming oncogene that has been reported to be involved in cell invasion and motility. High-density tissue microarray analysis shows that increasing PKC epsilon staining intensity is associated with high histological grade, positive Her2/neu receptor status, and negative estrogen and PR status. PKC epsilon is a validated target for RNAi anticancer therapy based on the demonstration of in vivo inhibition of metastases in animals with experimental tumors. Thus, PKC epsilon plays a critical and causative role in promoting an aggressive MBC phenotype and as a target for anticancer therapy.

5.5.4.26 Retinoblastoma Tumor Suppressor Gene as a Biomarker

Approximately two-thirds of women with breast cancer have estrogen receptor-positive breast cancer, in which tumor growth is regulated by the natural female hormone estrogen, which is known to promote the growth of most types of breast cancer. However, another gene, the retinoblastoma tumor suppressor (RB) gene, is functionally inactivated in the majority of human cancers and is aberrant in one-third of all breast cancers. RB regulates G1/S phase cell cycle progression and is a critical mediator of antiproliferative signaling. RB deficiency compromises the short-term cell cycle inhibition following cisplatin, ionizing radiation, and

antiestrogen therapy of breast cancer with drugs such as tamoxifen (Bosco et al. 2007). Specific analyses of an RB gene expression signature in human patients indicate that deregulation of this pathway is associated with early recurrence following tamoxifen monotherapy. Thus, because the RB pathway is a critical determinant of tumorigenic proliferation and differential therapeutic response, it may represent a critical basis for directing therapy in the treatment of breast cancer. The RB tumor suppressor can be used as a biomarker for how tumors will respond to antiestrogen therapy and could become the basis for deciding how patients with estrogen receptor-positive breast cancer are treated clinically.

This is a way to predict when antiestrogen drug therapies are inappropriate for patients with hormone-dependent breast cancer so that physicians can immediately begin treating the patient with alternative drugs that are more likely to succeed. However, comprehensive clinical research is needed before this new method for predicting the success of antiestrogen drugs is applied in daily patient care.

5.5.4.27 Riboflavin Carrier Protein

Riboflavin carrier protein (RCP) serves to transport riboflavin to where it's needed throughout the body. RCP is estrogen modulated and is a growth and development protein that is synthesized and secreted by the liver. RCP plays a central role in pregnancy and infant development by transporting riboflavin across the placenta to a fetus and from the mother to an infant. During pregnancy, RCP levels increase dramatically to support the needs of the fast growing cells. Serum RCP levels in cycling breast cancer patients are 3–4-fold higher than those in their normal counterparts. This difference in circulatory RCP levels between cancer patients and their age-matched normal counterparts is further magnified to 9–11-fold at the post-menopausal stage. In addition, there seems to be a good correlation between rising RCP levels and disease progression, since significantly higher RCP concentrations are encountered in patients with advanced metastasizing breast cancer versus those with early disease. Using specific MAbs, RCP can be localized immunohistochemically in the cytoplasm of invading neoplastic cells of lobular and ductal carcinomas of the breast, indicating that the malignant cells are probably the source of the elevated serum RCP levels in breast cancer. These findings suggest that measurement of circulatory RCP and the immunohistochemical staining pattern of RCP in biopsy specimens could be exploited as an additional biomarker in diagnosis/prognosis of breast cancer in women. The measurement of RCP can serve as a specific biomarker for women-related cancers in the determination of both presence and stage of the disease due to its estrogen-driven relationship. Measurements conducted using radioimmunoassay (RIA) analysis have provided a reliable, sensitive, and specific method to detect elevated levels of RCP in blood serum. The ELISA clinical diagnostic technique may well be suited for early detection of estrogen-related cancers by measuring RCP. This test is being developed commercially by Memory Dx LLC.

5.5.4.28 Risk of Invasive Cancer After Diagnosis of Ductal Carcinoma In Situ

Biomarkers can identify which women who were initially diagnosed with DCIS are at high or low risk of subsequent invasive cancer, whereas histopathology information cannot. A nested case-control study in a population-based cohort of women who were diagnosed with DCIS and treated by lumpectomy alone showed that lesions that were p16+COX-2+Ki67+ or those detected by palpation were statistically significantly associated with subsequent invasive cancer (Kerlikowske et al. 2010). Eight-year risk of subsequent DCIS was highest for women with DCIS lesions that had disease-free margins of 1 mm or greater combined with either ER(−) ERBB2(+)Ki67(+) or p16(+)COX-2(−)Ki67(+) status. The finding will allow women with DCIS to be more selective about their course of treatment and, potentially, avoid aggressive forms of treatment such as complete mastectomy or radiation.

5.5.4.29 Serum CA 15-3 as Biomarker of Prognosis in Advanced Breast Cancer

Locally advanced breast cancer represents a heterogeneous subgroup of breast cancer with an often dismal outcome. Identifying prognostic factors has acquired great significance for the selection of optimal treatment in individual patients. Multimodality treatment options include chemotherapy followed by surgery, chemotherapy, and radiotherapy with the addition of tamoxifen in hormone receptor-positive cases. Baseline serum levels of CEA and carbohydrate antigen (CA 15-3) have emerged as strong independent predictors of outcome in locally advanced breast cancer (Martinez-Trufero et al. 2005). High preoperative concentrations of CA 15-3 are associated with adverse patient outcome. These biomarkers can be added to other established prognostic factors such as postoperative nodal status, histological grade, and response to adjuvant chemotherapy. Although CA 15-3 is currently used for monitoring therapy in advanced breast cancer, preoperative CA 15-3 levels may be combined with existing prognostic factors for predicting outcome in patients with newly diagnosed breast cancer.

5.5.4.30 Suppressor of Deltex Protein

Suppressor of deltex protein (SDRP), a ubiquitin ligase, is a component of the notch signaling pathway, which plays a key role in regulating cell proliferation. The human homolog of SDRP (hSDRP), which is aberrantly expressed in ER-positive breast cancer cell lines, adds value as a biomarker for early diagnostic testing for breast cancer and has potential use for the stratification of breast cancer patient groups and improved targeting of the most appropriate treatment for each patient.

Metastatic disease may be more common and aggressive in patients with cellular dysregulation relating to hSDRP in primary tumors. It is a target for future therapeutic strategies for breast cancer management.

5.5.4.31 Tumor Microenvironment as Biomarker of Metastasis in Breast Cancer

Multiphoton-based intravital imaging has shown that invasive carcinoma cells in rat mammary tumors intravasate when associated with perivascular macrophages, identifying a potential tumor microenvironment of metastasis (TMEM). TMEM is defined as the tripartite arrangement of an invasive carcinoma cell, a macrophage, and an endothelial cell. In a case–control study of patients who developed MBC, TMEM density predicted the development of systemic, hematogenous metastases (Robinson et al. 2009). The ability of TMEM to predict distant metastasis was independent of lymph node status and other currently used prognostic factors. Quantitation of TMEM may be a useful new prognostic biomarker for breast cancer patients. This is the basis of a test for metastasis that most pathology laboratories can carry out. The test consists of a triple immunostain containing antibodies to the three cell types. A high number of TMEMs in a tissue sample means that the tumor is likely to metastasize or has already done so. This test could help physicians to precisely identify patients that should receive aggressive therapy and might spare many women at low risk for metastatic disease from undergoing unnecessary and potentially dangerous treatment.

5.5.4.32 Type III TGF-β Receptor as Regulator of Cancer Progression

The TGF-β signaling pathway has a complex role in regulating mammary carcinogenesis, and type III TGF-β receptor (TβRIII), a ubiquitously expressed TGF-β coreceptor, acts as a genetic switch that regulates breast cancer progression and metastasis (Dong et al. 2007). Early in cancer, the protein acts as a tumor suppressor, inhibiting the uncontrolled growth of cells. But as the cancer progresses, the protein switches sides and begins to promote the metastasis of cancer. Most human breast cancers lose TβRIII expression correlating with decreased TβRIII expression. TβRIII expression decreases during breast cancer progression, and low TβRIII levels predict decreased recurrence-free survival in breast cancer patients. Restoring TβRIII expression in breast cancer cells by administering the drug 5-azacytidine dramatically inhibits tumor invasion, angiogenesis, and metastasis. These results indicate that loss of TβRIII through allelic imbalance is a frequent genetic event during human breast cancer development that increases metastatic potential. If further studies confirm these findings, physicians could use the presence or absence of this receptor as a biomarker to identify women who should be treated more aggressively in an effort to eradicate their cancers before they spread. However, even the most aggressive chemotherapy treatments can leave behind errant cancer cells that

later regrow and metastasize. To overcome this problem, it ultimately may prove possible to restore the TβRIII receptors in women prior to their receiving chemotherapy in order to inhibit the cancer's propensity to spread. Further studies are investigating whether measuring the levels of the TβRIII in cells can serve as a guide to making treatment decisions among cancer patients.

The fact that the flow cytometry approach enables one to determine the quantitative expression of important prognostic markers in breast cancer cells opens up unexpected possibilities for broad application of this technology in clinical samples obtained from needle biopsies or surgical biopsies of patients with breast cancer or with suspicion of breast cancers.

5.5.4.33 Diagnostic Tests Based on Breast Cancer Genes

Approximately 5–10 % of cases of breast cancer are due to inheritance of a mutated copy of one of the two genes known as BRCA1 and BRCA2. The mutational spectra of BRCA1 and BRCA2 include many high penetrance, individually rare genomic rearrangements. Among patients with breast cancer and severe family histories of cancer who test negative (wild type) for BRCA1 and BRCA2, ~12 % can be expected to carry a large genomic deletion or duplication in one of these genes. It is recommended that effective methods for identifying these mutations should be made available to women at high risk. A third gene mutation, CHEK2, is linked to high rates of breast cancer, although it is not as important as the BRCA1 and BRCA2 mutations in indicating breast cancer risk (Weischer et al. 2007). However, women may benefit from screening for the mutation, which was found in 1 % of white, Northern European women. The study only included Danish women, leaving questions about its prevalence in black and Hispanic women unanswered. In the study, 0.5 % of Danish women had the mutation, and 12 % of them developed breast cancer, compared to 5 % of the women who did not carry the mutation. Women with the mutation who were over 60, overweight, and taking hormone replacement therapy had a 24 % chance of developing breast cancer in 10 years.

BRACAnalysis (Myriad Genetics Inc.), a test for hereditary breast and ovarian cancer, incorporates the most thorough full-sequence analysis for gene mutation detection on a broad commercial scale. Further information has been discovered and published on an additional type of mutation, known as a large rearrangement that has not been detectable by commercial DNA sequencing technologies, but only by laborious, manual research-based methods. Such rearrangements are responsible for a small percentage of changes in the two breast cancer genes. Myriad added a panel of five common rearrangements to its BRACAnalysis test, accounting for nearly half of the total occurrence of large rearrangements in the two genes. Because large rearrangements are quite rare, a woman meeting the commonly employed selection criteria for BRACAnalysis has less than 0.5 % risk of carrying one of the large rearrangement mutations. Myriad's BRACAnalysis Rearrangement Test (BART), an automated molecular diagnostic test in the BRACAnalysis family of products, detects rare large rearrangements of the DNA in the BRCA1 and BRCA2

genes and is performed for women with exceptionally high risk who have tested negative for sequence mutations and the common large rearrangements already included in Myriad's test.

EMSY, another gene for breast and ovarian cancer that explains the link between hereditary and "sporadic" forms of these cancers, maps to chromosome 11q13.5, a region known to be involved in breast and ovarian cancer. EMSY gene is amplified almost exclusively in sporadic breast cancer (13 %) and higher-grade ovarian cancer (17 %). In addition, EMSY amplification is associated with worse survival, particularly in node-negative breast cancer, suggesting that it may be of prognostic value. The remarkable clinical overlap between sporadic EMSY amplification and familial BRCA2 deletion implicates a BRCA2 pathway in sporadic breast and ovarian cancer.

SEQUENOM Inc. has identified novel genetic markers in four genes for susceptibility to breast cancer. Each gene has forms that increase or decrease the risk for developing breast tumors. The company's data indicate common combinations of its proprietary breast cancer markers increase the average risk of developing breast cancer by a factor of 2, present in approximately 11 % of the female population. Certain rare combinations are estimated to increase the disease risk by up to a factor of 5. The protective forms of the genes are present in ~13 % of the female population and are associated with a fivefold decreased risk of developing breast cancer compared to the general population. This biomarker panel was identified in SEQUENOM's discovery genetics program using unrelated patient and control subjects of European descent. The company has replicated the initial association for these biomarkers in an independent Australian cohort. Knowledge of genetic risk can enable early intervention or prophylactic treatment options to offset that risk.

5.5.4.34 Prognostic Role of Breast Cancer Genes

Three genes, homeobox 13 (HOXB13), interleukin-17B receptor (IL17BR), and CHDH, as well as the HOXB13:IL17BR ratio index in particular strongly predict clinical outcome in breast cancer patients receiving tamoxifen monotherapy. A tumor bank study demonstrated that HOXB13:IL17BR index is a strong independent prognostic factor for ER+ node-negative patients irrespective of tamoxifen therapy (Ma et al. 2006). As a result of this study, these two biomarkers serve as the foundation of the AviaraDx Breast Cancer Profiling Technology.

Activity of a gene, Dachshund (DACH1), which normally regulates eye development and development of other tissues, commandeers cancer-causing genes and returns them to normal. DACH1 inhibits the expression of the cyclin D1 gene, an oncogene that is overexpressed in about half of all breast cancers. Analysis of over 2,000 breast cancer patients has demonstrated that DACH1 correlates with tumor size, stage, and metastasis, with its expression greatly reduced in MBC cells, but increased nuclear DACH1 expression predicts improved patient survival (Wu et al. 2006). The average survival was almost 40 months longer in women in whom their breast cancer continued to express DACH1. DACH1 gene reverts the cancerous

phenotype, thus turning the cell back to a premalignant state, and it could be used as a prognostic marker for breast cancer. Other cell fate-determining genes are being examined in an attempt to identify new therapeutics for breast cancer and metastasis.

Researchers at Fox Chase Cancer Center (Philadelphia, PA) have identified an important gene, CEACAM6 (carcinoembryonic antigen-related cell adhesion molecule 6), which is involved in the spread of breast cancer that has developed resistance to long-term estrogen deprivation. The gene may prove to be a useful biomarker for predicting, which patients have the greatest risk of breast cancer recurrence so their physicians can offer the most appropriate treatment plan. The research focused on breast cancer cells that had grown resistant to aromatase inhibitors (AIs), antihormone drugs to shut down the enzyme aromatase, which lets the body produce estrogen outside the ovaries. These drugs represent one of the most effective forms of hormone therapy for postmenopausal women whose breast cancer tests positive for ERs, which means that estrogen in the body fuels the growth of cancer cells. Unfortunately, one of the drawbacks to extended use of an AI may be that some of the cancer cells develop resistance to the drug and are able to grow and spread independent of estrogen. Several AI-resistant breast cancer cell lines were developed in the laboratory and found to be very invasive compared to AI-sensitive breast cancer cells. Analyses of gene activity in these AI-resistant cells showed that they express high levels of genes associated with invasiveness and metastasis. However, this aggressive behavior could be reversed by using siRNAs to knock out the CEACAM6 gene. This gene might be an important biomarker for metastasis and a possible target for novel treatments for patients with MBC.

Breast cells expressing high levels of p16 and/or COX-2, when coupled with proliferation, go on to become basal-like invasive tumors. These particular biomarkers indicate an abrogated response to cellular stress; cells overexpressing them that continue to proliferate have bypassed pRb-mediated signals to senesce. In contrast, cells with high p16 and/or COX-2, but low proliferation, have an intact Rb checkpoint and senescent program and do not go on to become tumorigenic. These biomarkers can be measured years before tumors actually arise and thus can be used clinically to help dictate individualized treatment options for breast cancer.

5.5.4.35 Protein Biomarkers for Breast Cancer Prevention

Protein biomarkers suitable for the prevention of breast cancer must be extremely sensitive, easily detectable, and highly correlated with the disease. They should be expressed in the reversible phase of carcinogenesis. Among the large number of candidate tumor-associated proteins, those related to the estrogen/chorionic gonadotropin/insulin pathway seem to be of most interest because these can be causally implicated. They presumably are the first to express differently and are open to hormonal treatments. The biomarkers that give information on membrane receptor-modulated signal transduction should be considered as well. Up to now, only tamoxifen has shown some preventive activity, suggesting that the estrogen pathway

is useful indeed. Fenretinide and recombinant human chorionic gonadotropin (hCG) are also promising. But the financial requirements and the very long assessment periods largely prevent current research. Application of proteomics combined with bioinformatics can provide specific combinations of disease-related expression profiles that could identify high-risk groups with much more reliability and enable monitoring of preventive strategies.

5.5.4.36 Biomarkers to Evaluate Efficacy of Chemoprevention

Breast cancer chemoprevention studies are in progress with antiestrogens, retinoids, and other drugs on preclinical models and on women with increased risk of developing breast cancer. It is still not known whether the above agents are efficacious in individual patients and which are the most reliable biomarkers to be assessed for efficacy. Several short-term bioassays have been developed for testing efficacy in animal models of breast cancer that simulate the development and progression of human breast cancer. These studies predominantly employ molecular biomarkers related to cell cycle progression, apoptosis, and senescence. Tamoxifen has been widely used for treatment as well as prevention of breast cancer. Tamoxifen may differentially affect cell proliferation and apoptosis in mammary tumors, and the expression levels of cyclin D1 and cyclin E might also be considered potential intermediate biomarkers of response of mammary tumors to tamoxifen and possibly to other selective ER modulators. Other biomarkers are currently under investigation for assessment of the efficacy of various chemopreventive agents.

5.5.4.37 Biomarkers of Response to Chemotherapy of Breast Cancer

Biomarkers of prognosis of breast cancer treated with doxorubicin. A manganese superoxide dismutase (MnSOD) polymorphism is a novel biomarker for the therapeutic response to doxorubicin in breast cancer patients, whereas a Val16Ala polymorphism of MnSOD is indicative of patient survival. More specifically, patients undergoing doxorubicin combination therapy with Val/Val, Val/Ala, and Ala/Ala genotypes had 95.2 %, 79 %, and 45.5 % survival rates, respectively, in a case study of 70 unselected breast cancer patients at NCI. Carriers of the Ala/Ala genotype had a highly significantly poorer breast cancer-specific survival in a multivariate Cox regression analysis than carriers of the Val/Val genotype. This technology can be developed into an assay to screen for breast cancer patients who would be responsive to doxorubicin treatment. Further, as the MnSOD polymorphism is common in the population (15–20 % of patients have the Ala/Ala genotype), it is a common risk factor for doxorubicin therapy. This technology can potentially be utilized as a screening tool applicable for all cancer types treated with doxorubicin and for personalizing treatment. Future studies include determination of the mechanism by which the polymorphism modulates doxorubicin toxicity.

Decreased breast density as a biomarker of response to tamoxifen. Increased breast density on mammography is the leading risk factor for breast cancer, apart from age. The International Breast Intervention Study I (IBIS-I), a trial of tamoxifen for ER-positive breast cancer prevention conducted at the Cancer Research UK Centre for Epidemiology, Mathematics and Statistics in London showed that a reduction in breast density of at least 10 % may predict who benefits from the breast cancer preventive effects of tamoxifen. Those with reduced breast density after 12–18 months of treatment had a 52 % reduced risk of breast cancer. By contrast, those women who did not have a decrease in breast density had only an 8 % risk reduction.

Biomarkers to predict response or resistance to aromatase inhibitors. Aromatase inhibitors (AIs) have been established as a useful hormonal therapy in hormone receptor-expressing breast carcinoma. However, changes in tumor protein expression after exposure to AIs are not well understood. These changes may provide insight into how breast carcinomas respond or develop resistance against AIs and lead to the discovery of potential biomarkers to predict treatment responses. Among various protein biomarkers that were investigated, HSP70 demonstrated the most significant positive correlation with clinical response of the patients to AIs (Yiu et al. 2010).

GRP78 as a predictor for chemoresponsiveness. GRP78 (78-kDa glucose-regulated protein), induced in the tumor microenvironment, is widely used as an indicator of the unfolded protein response (UPR). In vitro studies suggest that GRP78 confers chemoresistance to topoisomerase inhibitors, such as doxorubicin, which is used for the treatment of breast cancer. In a retrospective study of breast cancer patients who were treated with Adriamycin, archival tumor specimens were analyzed, and the relationship of GRP78 expression level to "time to recurrence" (TTR), used as a surrogate biomarker for drug resistance, was examined (Lee et al. 2006). The data show that 67 % of the study subjects expressed high level of GRP78 in their tumors before the initiation of chemotherapy and suggest an association between GRP78 positivity and shorter TTR. The use of GRP78 as a predictor for chemoresponsiveness and the potential interaction of GRP78 and/or the UPR pathways with taxanes warrant larger studies.

5.5.4.38 Concluding Remarks and Future Prospects of Breast Cancer Biomarkers

Numerous biomarkers of breast cancer have been investigated, but few have shown practical usefulness in management of patients. There is a need to start consolidating the pathways and analyzing overlapping/cooperative natures of molecules from pathways. Potential usefulness of the cytoplasmic kinases and coactivators, which may act as coregulators in the action of ER, is likely to accelerate the development of the next generation of biomarkers for the surveillance, prognosis, and therapeutic decisions for cancer (Ohshiro and Kumar 2010).

5.5.5 Cervical Cancer Biomarkers

Cancer of the cervix is the second most common cancer in women. The mortality rates of cervical cancer could be drastically reduced by the implementation of population-wide cytological screening test. Screening for CIN is usually performed by Pap smear or cervicovaginal lavage. Identification of women with abnormal cervical smears permits early treatment of lesions, but the high rate of false-positive and false-negative results is a cause for concern. The oncogenic human papillomavirus (HPV) is the causal factor in the development of cervical cancer. The detection of the viral infection enables the identification of patients at risk; however, about 5–30 % of the normal female population harbors these viruses, and only very few of these develop clinically relevant lesions.

Hybrid Capture (HC) 2 assay (Digene Corp.) is used for molecular diagnosis of HPV. HC 2 assay has a greater sensitivity to detect CIN grade 3 or higher, and its specificity is comparable to an additional cytological test indicating atypical squamous cells of undetermined significance (ASCUS) or a more advanced lesion. Testing for high-risk HPV with the HC 2 test is useful in the detection of CIN grade 2/3 in low-grade CIN groups and in the selection of patients for colposcopy. HC 2 test is now provided as a primary tool to detect cervical cancer along with Pap smears rather than as a secondary test. A growing body of data now demonstrates the ability of HPV testing to identify women at high risk of cervical cancer more accurately than the most advanced type of Pap smear. Some cancer experts even recommend that HPV test should replace Pap smear as the first-line tool for cervical cancer screening, particularly in low-resource countries of the developing world. HPV screening that distinguishes HPV16 and HPV18 from other oncogenic HPV types may identify women at the greatest risk of developing cervical cancer.

In advanced preneoplastic lesions, HPV genomes are often integrated into cellular chromosomes. This leads to enhanced expression of the viral oncogenes. The detection of specific viral mRNA transcripts derived from integrated HPV genomes enables the identification of preneoplastic lesions with a particularly high risk for progression to invasive cancers (APOT-assay). These findings will enable establishment of highly sensitive but specific and cost-efficient new cancer early detection assays.

The activity of two viral oncogenes E6 and E7 initiates in a long-term process neoplastic transformation in few of the HPV-harboring cells. As consequence of the expression of E7, a cellular marker protein (p16) is increasingly expressed in dysplastic cells. Therefore, MAbs directed against p16 enable specific identification of dysplastic cells and derived invasive cancers in histological slides as well as cytological smears by CINtec Assay (MTM Laboratories). Correlating the cancer-specific antigen to the histology and cytology, CINtec will provide more detailed and precise information for cancer screening and diagnostic to the examining pathologist. Clinical study data have already shown very promising results in its application for the early detection of cervical cancer. InPath System (Molecular Diagnostics Inc.) uses a specific combination of protein biomarkers that illuminate and map abnormal cells and is a useful method of screening for cervical cancer.

Exfoliated cervical cells are used in cytology-based cancer screening and may also be a source for molecular biomarkers indicative of neoplastic changes in the underlying tissue. However, because of keratinization and terminal differentiation, it is not clear that these cells have an mRNA profile representative of cervical tissue and that the profile can distinguish the lesions targeted for early detection. Comparison of the transcription profiles from samples of normal exfoliated cells and cervical tissues using whole-genome microarray shows that the gene expression profile of exfoliated cervical cells partially represents that of tissue and is complex enough to provide potential differentiation between cancer and benign conditions. These findings encourage further exploration of gene expression using exfoliated cells to identify and validate applicable biomarkers.

Expression of hTERT mRNA and protein has been investigated in cervix cancer, CIN, and normal cervix. Upregulation of hTERT may play an important role in the development of CIN and cervix cancer; hTERT could be used as an early diagnostic biomarker for cervix cancer.

Several studies have been presented evaluating p16INK4a, a potential biomarker for cervical cancer screening and diagnosis. CINtec p16INK4a-based immunocytochemistry protocols have been used on cervical cytology preparations. The results of the studies indicate that this approach improves the histological diagnosis of cervical cancer.

5.5.6 Gastrointestinal Cancer Biomarkers

Three important cancers of the gastrointestinal system are esophageal cancer, gastric cancer, and CRC. These will be described with regard to biomarker studies.

5.5.6.1 Esophageal Cancer Biomarkers

Carcinoma of the esophagus including carcinoma of gastroesophageal junction is rapidly increasing in incidence. Esophageal carcinogenesis is a multistage process, involving a variety of changes in gene expression and structure. Identification of dysplasia in mucosal biopsies is the most reliable pathological indicator of an increased risk of development of squamous cell carcinoma and passes through the sequence of chronic esophagitis, low-grade and high-grade dysplasia, and invasive carcinoma. Barrett's esophagus is a precursor to esophageal adenocarcinoma (EAC) and has a well-described sequence of carcinogenesis: the Barrett's metaplasia–dysplasia–adenocarcinoma sequence. Studies are in progress to discover biomarkers for risk of squamous cell carcinoma as well as the diagnosis and monitoring of the response to treatment.

Hypermethylation of several tumor suppressor genes is involved in the evolution and progression of EACs. Efforts are now underway to develop noninvasive biomarkers for this disease. Hypermethylation of APC gene occurs in the plasma of

25 % of EAC patients, and this is significantly associated with reduced patient survival, suggesting that APC hypermethylation in the plasma may be a useful biomarker of biologically aggressive disease in EAC. Similarly 23 % of EAC patients have hypermethylation of p16 gene in the serum DNA. Various studies have found the usefulness of analyzing methylation levels of p16, E-cad, RARb, death-associated protein kinase (DAPK), and APC in the peripheral blood not only as a screening and monitoring tool for EAC patients but also as a biomarker of tumor recurrence (Ikoma et al. 2007; Hoffmann et al. 2009).

miRNA expression profiles of esophageal cancer reveal the oncogenic mechanism by miRNA-mediated posttranscriptional pathway. Further exploration is required for better understanding their role in carcinogenesis of esophageal cancer. Circulating miRNAs are potential biomarkers for esophageal cancer (Zhou and Wang 2010).

5.5.6.2 Gastric Cancer Biomarkers

While there is no reliable serum biomarker for the diagnosis and monitoring of patients with gastric cancer, proteomic technologies have been used extensively for detection of biomarkers of gastric cancer. An analysis of cryostat sections of central gastric tumor, tumor margin, and normal gastric epithelium using ProteinChip Arrays and SELDI-TOF MS revealed a peak that was significantly downregulated in tumor tissue and identified as pepsinogen C using MS/MS analysis and immunodepletion (Melle et al. 2005). This signal was further characterized by IHC. This work demonstrates that differentially expressed signals can be identified and assessed using a proteomic approach comprising tissue microdissection, protein profiling, and IHC. Pepsinogen C is a potential biomarker of gastric cancer.

Serum samples from patients with gastric cancer as well as healthy adults were examined by SELDI-TOF MS, and data of spectra were analyzed by Biomarker Patterns Software (Qian et al. 2005). Two mass peaks were selected as significant potential biomarkers. The sensitivity, specificity, and accuracy of the model were higher than those of clinically used serum biomarkers CEA, CA 19-9, and CA72-4. Stage I/II gastric cancer samples of the test group were all judged correctly. The novel biomarkers in serum and the established model could be potentially used in the detection of gastric cancer. However, large-scale studies should be carried on to further explore the clinical impact on the model.

Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) is overexpressed in many human malignancies, including gastric cancer, and is associated with poor outcome. An elevated preoperative level of serum TIMP-1 was significantly associated with progressive disease, advanced stage, and worse survival in gastric cancer patients who underwent surgery (Wang et al. 2006).

Aberrant DNA methylation is an early and frequent process in gastric carcinogenesis and could be useful for detection of gastric cancer. Six genes (MINT25, RORA, GDNF, ADAM23, PRDM5, MLF1) showed frequent differential methylation between gastric cancer and normal mucosa with close correlation between

methylation levels in tumor biopsy and gastric washes (Watanabe et al. 2009). Of these, MINT25 was found to be a sensitive and specific biomarker for screening in gastric cancer. Other genes that have been studied as biomarkers of gastric cancer using MSP include APC, DAPK, E-cad, GSTP1, MGMT, MLH1, MSP, p15, p16, Q-MSP, RUNX3, RARb, RASSF1A, SOCS1, TGFBR2, and TIMP3.

Real-time RT-PCR has shown that expression levels of miR-106a and miR-17 in preoperative and postoperative blood samples of patient with gastric carcinoma were significantly higher than those in controls, indicating that this may be a new tool for monitoring CTCs (Zhou et al. 2010). In a similar study, plasma analyses for five selected miRNAs (miR-17-5p, miR-21, miR-106a, miR-106b, and let-7a) were performed in gastric cancer patients and healthy volunteers (Tsujiura et al. 2010). The miRNAs were stable and detectable in all plasma samples, and the plasma miRNA levels reflected the tumor miRNAs in most cases. The levels of these miRNAs were significantly reduced in postoperative samples, indicating the prognostic value of miRNAs in gastric neoplasia. In large-scale analysis, the plasma concentrations of four of the five miRNAs (miR-17-5p, miR-21, miR-106a, and miR-106b) were significantly higher in cancer patients than in controls, whereas mir-let-7a was lower in cancer patients. These results support the specificity and usefulness of this method detecting circulating miRNAs for diagnosis as well as prognosis.

5.5.6.3 Colorectal Cancer Biomarkers

CRC is one of the most common cancers in the world and is a leading cause of cancer mortality and morbidity. The cause of CRC is multifactorial, involving hereditary susceptibility, environmental factors, and somatic genetic changes during tumor progression. Detection of biomarkers is useful for prevention, diagnosis, prognosis, and management of CRC. Biomarkers of CRC are listed in Table 5.7.

Detection of serum biomarkers of CRC. One method for detecting serum biomarkers for CRC is by serum protein profiling using SELDI-TOF MS followed by classification tree pattern analysis. Biomarkers can be identified and reproducibly detected in independent sample sets with high sensitivity and specificity. Although not specific for CRC, these biomarkers have a potential role in monitoring the disease as well as the treatment. However, there is still a need for multiple biomarker testing and for identifying panels of predictive markers in order to improve response rates and decrease toxicity with the ultimate aim of tailoring treatment according to an individual patient and tumor profile. Soluble cytokeratin-18 fragment M65A is released from human cancer cells during cell death and is a potential biomarker of CRC that is characterized by frequent metastatic spread (Ausch et al. 2009).

BioServe and Phenomenome Discoveries Inc. (PDI) have developed a novel serum-based diagnostic test for the identification of CRC and precancerous states conducive to the development of CRC. For developing the test, BioServe identified a large number of patient tissue and serum samples from its Global Repository exhibiting CRC across a spectrum of stages, as well as matched healthy controls.

Table 5.7 Biomarkers of colorectal cancer

Aldo-keto reductase family 1 B10 (AKR1B10 or ARL-1)
Alpha-methylacyl-coenzyme A racemase (AMACR) in CRC tissue as prognostic biomarker
Biomarkers of resistance to chemotherapy: thymidylate synthase, topoisomerase-I, ↑ABCB1/P-gp transporter
CRC-specific methylated DNA biomarkers in plasma: MLH1, p16, DAPK, TPEF/HPP1, APC, HLTF, ALX4, RUNX3, RASSF1A, RASSF2A, SEPT9, MGMT, and WIF1
Circulating tumor cell biomarkers: plastin3
Desmin
DNA microsatellite instability
Gene biomarkers of CRC
Guanylyl cyclase C
Insulin and insulin-like growth factor binding protein (IGFBP)-1
Matrix metalloproteinase 9
miRNA biomarkers of CRC
Mutations in DNA mismatch repair genes: hereditary nonpolyposis CRC
Serum CEA
Urinary biomarkers
Volatile organic compounds in exhaled breath

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Using PDI's patented nontargeted metabolomic platform, PDI discovered that a series of novel metabolites were significantly decreased in serum samples collected from CRC patients compared to controls. From these results, PDI developed a 2 min high-throughput screening method capable of simultaneously measuring a key subset of these molecules. The rapid test was found to have a sensitivity of 75 % and specificity of 90 %. Clinical trials are planned to evaluate the test's utility as part of a broad-based population screening regimen.

Circulating tumor cell biomarkers. CTCs in blood are potential seeds for metastasis as well as cancer biomarkers. However, most CTC detection systems might miss EMT-induced metastatic cells because detection is based on epithelial markers. Microarray analysis of CRC tissue specimens to detect genes that are overexpressed relative to normal colon mucosa led to discovery of plastin3 (PLS3) as a biomarker that is expressed in metastatic CRC cells but not in normal circulation (Yokobori et al. 2013). Fluorescent immunocytochemistry was used to validate that PLS3 was expressed in EMT-induced CTC in peripheral blood from patients with CRC with distant metastasis. PLS3 has also significant prognostic value.

Urinary biomarkers of CRC. Metabolomic research remains the primary means to identify urinary biomarkers in CRC. However, many serum biomarkers and tissue biomarkers are not excreted in the urine unless the plasma levels are high enough to overcome renal resorption. According to one study, 76.9 % of CRC patients can be correctly classified using principal component analysis of 14 nucleosides by reversed-phase HPLC; 9 of these nucleosides were found to significantly decrease after curative resection thus implying prognostic value (Feng et al. 2009). This preliminary study indicates that the evaluation of both normal and

modified urinary nucleosides might provide unique markers in the diagnosis and management of CRC.

miRNA biomarkers of CRC. CRC samples, characterized by microsatellite stability (MSS) as well as high microsatellite instability (MSI-H), have been investigated for genome-wide expression of miRNA and mRNA (Lanza et al. 2007). Based on combined miRNA and mRNA gene expression, a molecular signature consisting of differentially expressed genes including miRNAs, could correctly distinguish MSI-H versus MSS colon cancer samples. Among the differentially expressed miRNAs, various members of the oncogenic miR-17-92 family were significantly upregulated in MSS cancers. The majority of protein-coding genes were also upregulated in MSS cancers. Their functional classification revealed that they were most frequently associated with cell cycle, DNA replication, recombination, repair, gastrointestinal disease, and immune response. This study suggests that the combination of mRNA/miRNA expression signatures may represent a general approach for improving biomolecular classification of human cancer. In 2010, Rosetta Genomics plans to launch miRscreen Colon, which shows that the expression patterns of two miRNAs can be used to identify the presence of the disease with 91 % sensitivity and 72 % specificity.

Some of the miRNAs may function as oncogenes due to their overexpression in tumors; hsa-miR-200c may be a potential novel prognostic factor in CRC. Another study has shown that altered expression levels of miR-21, miR-31, miR-143, and miR-145 is associated with clinicopathologic features of CRC (Slaby et al. 2008).

Differential expression of specific miRNAs in tissues and blood offers the prospect of their use in early detection, screening, and surveillance of CRC in a noninvasive manner. A study investigated whether plasma miRNAs could discriminate between patients with and without CRC (Ng et al. 2009). This study entailed an initial discovery of discriminatory miRNAs in a small subset of CRC patients versus normal subjects, followed by selection and validation of these miRNAs in an independent collection of plasma from patients with CRC, gastric cancer, and inflammatory bowel disease as well as healthy controls. Among the panel of 95 miRNAs analyzed, 5 miRNAs (miR-17-3p, miR-135b, miR-222, miR-92, and miR-95) were upregulated both in plasma and tissue samples. All five miRNAs were validated in the plasma of patients with CRC and healthy controls. Among these, two miRNAs, miR-17-3p and miR-92, were significantly elevated in the patients with CRC. The plasma levels of these two miRNAs were significantly reduced after surgery in patients with CRC. Further validation with an independent set of plasma samples indicated that miR-92 differentiated CRC from gastric cancer, IBD, and normal subjects.

ColonSentry (GeneNews) is based on the Sentinel Principle™, which uses blood samples to identify RNA biomarkers for early diagnosis of CRC. It has been developed to provide:

- A simply convenient first step for CRC screening
- High patient acceptance
- To exploit genomics for CRC diagnostics
- To enhance the number of cancers detected by colonoscopy

Hereditary nonpolyposis colorectal cancer (*HNPCC*). This is a familial cancer syndrome characterized by mutations in at least one of six DNA mismatch repair genes: hPMS1, hPMS2, hMSH2, MSH6, hTGFBR2, and hMLH1. Five to 10 % of the 150,000 cases of CRC diagnosed each year in the USA are of hereditary type. Identification of DNA microsatellite instability refines the diagnosis of HNPCC, allowing frequent early-onset colonoscopic screening to be restricted to individuals with an especially high risk of this type of cancer. Human homologs of murine miRNA sequences, miR-143 and miR-145, consistently display reduced steady-state levels of the mature miRNA at the adenomatous and cancer stages of colorectal neoplasia and are potential biomarkers.

Diagnostic biomarkers of CRC. Increased level of matrix metalloproteinase 9 (MMP-9), a biomarker of CRC that can be measured from a blood sample, is potentially an accurate, low-risk, and cost-effective population screening tool. The accuracy of serum MMP-9 as a test for CRC in a primary care population is being evaluated.

Epigenomics Inc. is using a sieving strategy for identifying high-performing biomarker assays that detect CRC-specific methylated DNA in plasma (Lofton-Day et al. 2008). Restriction enzyme-based discovery methods were used to identify biomarker candidates with obviously different methylation patterns in CRC tissue and nonpathological tissue. A selection process incorporating microarrays and/or real-time PCR analysis of tissue samples was used to further test biomarker candidates for maximum methylation in CRC tissue and minimum amplification in tissues from both healthy individuals and patients with other diseases. Three biomarkers, TMEFF2, NGFR, and SEPT9, were selected and tested with plasma samples. TMEFF2 methylation was detected in 65 % of plasma samples from CRC patients and not detected in 69 % of the controls. The corresponding results for NGFR were 51 and 84 %; for SEPT9, the values were 69 and 86 %. Application of stringent criteria at all steps of the selection and validation process enabled successful identification and ranking of blood-based biomarker candidates for CRC.

A novel blood-based, five-gene biomarker set has been reported for the detection of CRC (Han et al. 2008). Two of these were the most upregulated (CDA and MGC20553), and three were the most downregulated (BANK1, BCNP1, and MS4A1) in CRC patients. The predictive power of these five genes was validated with a novel third set, correctly identifying 88 % of CRC samples and 64 % of non-CRC samples.

Guanylyl cyclase C (GCC) is a cell surface molecule found on colorectal cells, both normal and cancerous, but not on any normal cells outside the intestine. GCC receptor provides a superior mechanism for detecting the presence of CRC cells because it relies on ultrasensitive mRNA-based amplification technology rather than other less sensitive and variable detection systems, such as the histopathology. Quantification of GCC mRNA in tissues by RT-PCR employing external calibration standards is analytically robust and reproducible, with high clinicopathologic sensitivity and specificity (Schulz et al. 2006). GCC biomarker has shown to be 95–100 % accurate in detecting the spread or recurrence of colon cancer in lymph nodes or blood. This is the basis for Previstage™ GCC (DiagnoCure) as a lymph node test for the staging of CRC.

The aldo-keto reductase family 1 B10 (AKR1B10 or ARL-1) protein is normally expressed in mature epithelial cells of healthy colon tissue. AKR1B10 expression is noticeably decreased or absent in CRC and precancerous conditions. AKR1B10 can be used as a novel biomarker for CRC and other precancerous conditions. It would be useful in screening high-risk populations, such as those with predisposing conditions of CRC, such as Crohn's disease, chronic inflammatory bowel disease, and ulcerative colitis.

Desmin, originally a tissue biomarker of heart failure, is found to be elevated in CRC tissue and fetal colorectal tissue compared with normal colorectal tissue. Desmin can be considered a potential serum biomarker for CRC that may have significance in the detection of patients with CRC (Ma et al. 2009).

Analysis of the VOCs linked to cancer is a new frontier in cancer screening, as tumor growth involves several metabolic changes leading to the production of specific compounds that can be detected in exhaled breath. Canine scent detection has shown that odor of VOCs is an effective tool in CRC screening. A study investigated whether patients with CRC have a specific VOC pattern compared with the healthy population (Altomare et al. 2013). Exhaled breath was collected in an inert bag (Tedlar®) from patients with CRC and healthy controls (negative colonoscopy) and processed offline by thermal-desorber gas chromatography–MS to evaluate the VOC profile. During the trial phase, VOCs of interest were identified and selected, and VOC patterns able to discriminate patients from controls were set up; in the validation phase, their discriminant performance was tested on blinded samples. A probabilistic neural network (PNN) validated by the leave-one-out method was used to identify the pattern of VOCs that better discriminated between the two groups. Application of a PNN to a pattern of 15 compounds showed a discriminant performance with a sensitivity of 86 %, a specificity of 83 %, and an overall accuracy of 76 %. The pattern of VOCs in patients with CRC was different from that in healthy controls. Breath VOC analysis appears to have potential clinical application as a biomarker in CRC screening, although further studies are required to confirm its reliability in heterogeneous clinical settings.

Biomarkers for prevention and management of CRC. A study team at Emory University (Atlanta, GA) is analyzing the rectal tissue samples of people with colon adenomatous polyps and comparing them to rectal tissue samples from people who do not have polyps to discover biomarkers that may predict risk of developing CRC. The researchers are also looking at whether the differences they detect in rectal tissue can also be found in blood or urine. The team is also part of a 10-year multisite US study examining whether increased consumption of vitamin D and calcium acts on biomarkers of risk for colon cancer and prevents the recurrence of adenomatous polyps. In a case–control study, nested within the European Prospective Investigation into Cancer and Nutrition, no interactions were noted for any of the polymorphisms with serum 25OHD concentration or level of dietary calcium confirming a role for the BsmI polymorphism of the VDR gene in CRC risk, independent of serum 25OHD concentration and dietary calcium intake (Jenab et al. 2009).

It is possible that a combination of tests for microsatellite instability, allelic loss, p53 mutations, and other genetic alterations in patients with early-stage CRC will define groups of patients who require different adjuvant therapies or no systemic treatment at all. Serum CEA has also been used as a biomarker for surveillance and monitoring of therapy of CRC. Despite the recent encouraging data, the clinical use of targeted therapy is hampered by several questions that need to be answered such as optimal biological dose and schedule, lack of predictive surrogate biomarkers, and modalities of combination with chemotherapy/radiotherapy. To improve this situation, high-throughput methods have been used to discover prognostic and predictive markers for CRC.

Biomarkers of survival in CRC. Use of AQUA™ (HistoRx) technology that combines fluorescence-based imaging, microscopy, and high-throughput tissue microarray technologies has shown that the location and amount of thymidylate synthase (TS) within two separate compartments of a tumor cell may be critical biomarkers for predicting survival in CRC. High levels of the protein in the nucleus correlate with decreased patient survival time, and, further, a high ratio of TS in the nucleus relative to the level in the cytoplasm correlates with a shorter survival time.

Obesity, sedentary lifestyle, and Western dietary pattern have been linked to increased risk of cancer recurrence and mortality among patients with surgically resected CRC. Excess energy balance leads to increased circulating insulin and depressed levels of circulating insulin-like growth factor-binding protein (IGFBP)-1, which promote cancer cell growth in preclinical models. Thus, circulating insulin and IGFBP-1 are potential mediators of the association between lifestyle factors and mortality after CRC resection. Blood levels of these two insulin-related proteins can be used as biomarkers to predict which patients with CRC are most likely to die of their disease. In a study on patients with surgically resected CRC, higher levels of prediagnosis plasma C-peptide and lower levels of prediagnosis plasma IGFBP-1 were associated with increased mortality (Wolpin et al. 2009). Those with the highest levels of plasma C-peptide had an 87 % greater chance of dying overall and a 50 % greater chance of dying from CRC than those with the lowest levels. The difference may be due to the fact that C-peptide is basically insulin, which is associated with diseases of the heart and other systems.

Men consuming high amounts of red meat and dairy products are at a higher risk of developing colon and prostate cancer. Alpha-methylacyl-coenzyme A racemase (AMACR) is an enzyme that helps to break down fat from these foods to produce energy. An increase in the utilization of energy from fat is a hallmark of many cancers. AMACR is also highly expressed in certain stages of CRC, and a close examination of the this gene in a panel of normal and progressively malignant colon tissues reveals that deletions of specific sequences in the AMACR gene may trigger its abnormal expression during the evolution of CRC (Zhang et al. 2009). A new deletion variant of the AMACR gene may serve as a biomarker of prognosis and survival in CRC.

Biomarkers of resistance to chemotherapy. Despite the recent results of systemic chemotherapy, more than 40 % of patients with advanced cancer still do not achieve substantial benefits with cytotoxic agents. Resistance to chemotherapy is an important factor in poor response to treatment. Mechanisms that may have important implications for drug efficacy and actively contribute to innate resistance in CRC are as follows:

- High levels of TS, the 5-FU target, are associated with tumor insensitivity to FU-based therapy.
- Higher levels of topoisomerase-I (TOP1) correlate with greater sensitivity of colon tumors to camptothecin derivatives compared to normal colonic mucosa.
- Glucuronidation, involved in xenobiotic detoxification, is also associated with innate resistance to TOP1 inhibitors in colon cell lines and tumors.
- An increase of the ABCB1/P-gp transporter, a member of the family of ABC-transporters that detect and eject anticancer drugs from cells, is observed in intrinsically drug-resistant colon tumors.

The success of chemotherapy depends on various factors such as gender, age, and histological subtype of tumor. The difference in drug effects between different genotypes can be significant. Promising candidates have been identified with predictive value for response and toxicity to chemotherapy in CRC. These candidates need to be incorporated into large, prospective clinical trials to confirm their impact for response and survival to chemotherapy that has been reported in retrospective analyses. Confirmed predictive markers, together with additional yet to be identified pharmacogenomic key players, will provide the basis for tailoring chemotherapy in the future. The rationale for this approach is based on the identification of the in vivo interactions among patient's characteristics, disease physiopathology, and drug pharmacodynamics as well as pharmacokinetics.

Biomarkers for personalized management of CRC. In 2011, OncoTrack, an international consortium of academic researchers, pharmaceutical companies, and commercial partners, launched a 5-year project to develop and assess new biomarkers for CRC. OncoTrack was founded to create next-generation methods of biomarker development to develop personalized treatment of CRC. The consortium's first project called "Methods for systematic next-generation oncology biomarker development" will seek to generate high-quality genomic and epigenetic data from clinically well-defined CRC tumors and their metastases. The data will be compared to the germline genome of the patients and will be complemented by a detailed molecular characterization of the tumors. OncoTrack will establish and characterize a new series of xenograft tumor models and cell lines derived from the same set of tumors in order to support tumor biology research and the early stages of biomarker qualification. The combined data from all phases of the project will enable OncoTrack to address fundamental questions regarding the relationship between tumor genotype and phenotype, thus providing the starting point for discovery and selection of suitable candidates for development as biomarkers of CRC.

5.5.7 Head and Neck Cancer

HNSCC is a leading cause of cancer mortality worldwide. Gene expression signatures generated from DNA microarray analyses using formalin-fixed HNSCC tumors have shown that genes involved in EMT and nuclear factor-kappaB (NF-κB) signaling deregulation are the most prominent molecular characteristics of the high-risk tumors (Chung et al. 2006a). The difference in recurrence-free survival between the high-risk versus low-risk groups was statistically significant. The 75-gene list, determined by training on the formalin-fixed tumor dataset and tested on data from the independent frozen tumor set, can be used as a prognostic biomarker of recurrence. These data suggest that the molecular determinants of EMT and NF-κB activation can be targeted as the novel therapy in the identified high-risk patients.

Recent reports have associated a subset of HNSCC with high-risk HPVs, particularly HPV16, the same subset of HPVs responsible for the majority of cervical and anogenital cancers. In a transgenic mouse model for HPV-associated HNSCC, HPV16 oncogenes mirror the molecular and histopathological characteristics of human HPV-positive HNSCC that distinguish the latter from human HPV-negative HNSCC (Strati et al. 2006). This validated model provides the means to define the contributions of individual HPV oncogenes to HNSCC and to understand the molecular basis for the differing clinical manifestations of HPV-positive and HPV-negative human HNSCC. This study identified minichromosome maintenance protein 7 (MCM7) and p16 as potentially useful biomarkers for HPV-positive head and neck cancer. However, other studies have shown that a positive test for HPV DNA alone is not significantly linked to head and neck cancer outcomes. On the other hand, when found in combination with E6 and E7 expression, a positive HPV16 test did coincide with improved oropharyngeal cancer outcomes. Likewise, elevated levels of p16 in a tumor were not especially informative on their own, though they do correspond to better oropharyngeal cancer survival when found together with positive blood tests for E6 and E7. Another study on oropharyngeal squamous cell carcinomas (OPSCC) found its own evidence arguing against the use of HPV DNA as a solo biomarker for HPV-associated cancer (Holzinger et al. 2012).

The proto-oncogene pituitary tumor-transforming gene (PTTG) has been shown to be abundantly overexpressed in a large variety of neoplasms likely promoting neovascularization and tumor invasiveness. Elevated PTTG transcript levels might be used as a prognostic biomarker for future clinical outcome (i.e., recurrence) in primary squamous cell carcinomas of the head and neck, especially in early stages of tumor development.

EGFR is another promising biomarker for HNSCC, but further research is required to determine its prognostic benefit. Several promising biomarker candidates are now being evaluated, including epigenetic-, expression-, and genomic-based biomarkers. Studies to validate the sensitivity and specificity of these biomarkers in clinical samples from adequately powered prospective cohorts are needed for successful translation of these findings into potential molecular diagnostic, prognostic, and therapeutic biomarkers for HNSCC (Mydlarz et al. 2010).

A close look at the HNSCC transcriptome analyses has revealed some genes that are frequently dysregulated and are specific candidates as HNSCC molecular biomarkers (Lallemant et al. 2009). Nine genes displaying frequent alterations in HNSCC are FN1, MMP1, PLAU, SPARC, IL1RN, KRT4, KRT13, MAL, and TGM3. MMP1 detection in saliva rinse is potentially useful for noninvasive diagnosis of HNSCC of the oral cavity or oropharynx with 100 % specificity, but technical improvement is needed since sensitivity is only 20 %. IL1RN, MAL, and MMP1 are prospective tumor diagnostic biomarkers for HNSCC. MMP1 overexpression is the most promising biomarker, and its detection could help identify tumor cells in tissue or saliva.

5.5.8 Leukemia Biomarkers

Conventional methods for the diagnosis of leukemias are blood counts with staining and examination of cells, examination of bone marrow following aspiration, biochemical screening, chromosome analysis of cells to detect dislocations, and immunophenotyping. Molecular diagnostics is now applied for assessment of leukemias. Various biomarkers of leukemias are described here fall into the following categories:

- Chromosome translocations in leukemias
- Gene mutations
- Proteins
- miRNA biomarkers

5.5.8.1 Chromosome Translocations in Leukemias

Chromosome translocations (rearrangements), which are present in most human leukemias, are widely used by clinicians as diagnostic and prognostic tools. At the molecular level, translocations are especially valuable because they immediately indicate the spot in which to search for a cancer gene. Chromosome breakpoints can now be cloned and sequenced efficiently, and the relevant genes can be rapidly identified. The t(9;22) translocation known as the Philadelphia chromosome was the first tumor-specific cytogenetic marker identified in a human cancer. Its discovery eventually led to the cloning of the BCR–ABL fusion region. The presence of a Philadelphia chromosome confers a poor prognosis in cases of ALL. Rearrangements of the MLL (mixed lineage leukemia) gene located at chromosome band 11q23 are commonly involved in adult and pediatric cases of primary acute leukemias and also found in cases of therapy-related secondary leukemias. Approximately 50 different chromosomal translocations of the human MLL gene are currently known and associated with high-risk acute leukemia. The large number of different MLL translocation partner genes makes a precise diagnosis a demanding task. After their

cytogenetic identification, only the most common MLL translocations are investigated by RT-PCR analyses, whereas infrequent or unknown MLL translocations are excluded from further analyses. A universal long-distance inverse-PCR approach enables the identification of any kind of MLL rearrangement if located within the breakpoint cluster region including previously unrecognized partner genes. Furthermore, the determined patient-specific fusion sequences are useful for minimal residual disease monitoring of MLL-associated acute leukemias.

Various specific chromosome rearrangements, including t(8;21), t(15;17), and inv(16), are found in acute myeloid leukemia (AML), and in childhood acute lymphocytic leukemia (ALL), t(12;21), and t(1;19) are common. Most childhood leukemias begin before birth and that maternal and perinatal exposures such as chemical and infectious agents are likely to be critical. Epigenetic events are also important in the development of some forms of childhood leukemia. Some studies now show that the inactivating NAD(P)H:quinone acceptor oxidoreductase (NQO1) C609T polymorphism is positively associated with leukemias arising in the first 1–2 years of life and polymorphisms in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene have been associated with adult and childhood ALL.

5.5.8.2 DNA Methylation Biomarkers in Leukemia

Several studies have shown that methylated DNA biomarkers can be used to detect leukemia and other hematological malignancies. Histone H3 methylation may be changed dramatically during normal cell differentiation. Residual histone H3 methylation in myeloid leukemia cells suggests an incomplete chromatin condensation that may be linked to the leukemia cell proliferation and may be important for the prognosis of disease. Advantages of the study of methylation biomarkers in leukemia are the following:

- Sensitivity of detection of DNA in blood eliminates the need for tissue biopsy.
- Ease of use of DNA for clinical assay.
- Cost-effective as it can replace the need to perform medical imaging or tissue biopsy.
- Multiple uses: diagnosis, monitoring of treatment, and detection of disease relapse.

5.5.8.3 Gene Mutations as Biomarkers in Leukemia

Patients with AML and normal karyotype constitute the single largest cytogenetic group of AML, estimated to account for 45 % of adults with de novo AML. Four prognostic biomarkers can predict outcome in patients with AML and normal cytogenetics: (1) internal tandem duplication and point mutations in the FLT3 gene, (2) partial tandem duplication of the MLL gene, (3) mutations of the CEBPA gene, and (4) overexpression of the BAALC gene. Because mutations in FLT3 result in an

autophosphorylated, leukemogenesis-driving protein, molecular-targeting therapy with a new class of tyrosine kinase inhibitors is being explored in early clinical trials. Considerable progress has been made in molecular characterization of AML patients with normal cytogenetics. The challenge for the future is to incorporate these biomarker discoveries into novel risk-adapted therapeutic strategies that will improve the currently disappointing cure rate (~25 to 40 %) of this group of patients.

Genzyme Diagnostics has launched two molecular tests for AML: FLT3 Mutation Analysis and WT1 RQ-PCR. FLT3 mutations are considered a prognostic indicator of poor survival and response to standard chemotherapies as ~30 % of patients with AML have FLT3 mutations. WT1 RQ-PCR test is designed to detect MRD or very low levels of disease. The WT1 gene is expressed in ~90 % of patients with AML. This test enables physicians to monitor AML patients for early relapse during and following therapy. Both of these tests may enable oncologists to better manage their patients.

5.5.8.4 Molecular Diagnostic Techniques for Leukemia

Molecular probes are used to diagnose acute or chronic leukemia. Both DNA mapping by Southern blotting and PCR can be used to detect chromosomal translocations, e.g., the Philadelphia chromosome, and determine the type of rearrangement. BCR–ABL translocation can be detected and quantified with the use of mRNA down to a level of 10^{-6} (i.e., 1 leukemia cell per 10^6 total cells). Light microscopy, cytogenetic analysis, flow cytometry, ISH, and Southern blot analysis will not detect malignant cells until their population exceeds 1–5 % of the normal cell total. By contrast, PCR is sufficiently sensitive to detect 1 leukemia cell among 100,000 or even one million normal cells.

FISH probes directed against the BCR and ABL genes can reliably detect the fusion gene with a sensitivity of 0.05 %. RT-PCR is capable of detecting very low levels of BCR–ABL mRNA transcripts allowing detection of a single leukemia cell. Real-time PCR can quantify changes in transcript number and thus levels of residual disease and is useful in guiding clinical decision-making. This is particularly useful in detecting relapse following allogeneic stem cell transplantation and in predicting the likelihood of a durable complete cytogenetic response to interferon. Patients who become 100 % Ph negative on interferon, as detected by routine cytogenetic evaluation of 20 cells (20 % or less of interferon-treated patients), have a wide range of levels of PCR positivity. Only those with low levels of BCR–ABL transcripts will remain cytogenetically negative for prolonged periods.

Infants and children diagnosed with chemotherapy-resistant ALL may in fact have a different type of leukemia. Gene chip technology has been used successfully to categorize 95 % of the leukemias as ALL, AML, or MLL. The signatures provided by RNA profiling might have enough information content to enable not only to determine prognosis but to facilitate stratification of patients for personalized therapy.

5.5.8.5 Proteomic Technologies for Discovering Biomarkers of Leukemia

Proteomics is being used to subclassify leukemia, because cytogenetic analysis is costly and time-consuming. Several proteins have been identified that may serve as useful biomarkers for rapidly identifying different forms of childhood leukemia. Proteomic analysis of different subtypes of AML cells, carried out using 2D GE and MALDI-TOF analysis, can identify more significantly altered proteins that belong to the categories of suppressor genes, metabolic enzymes, antioxidants, structural proteins, and signal transduction mediators (Lopez-Pedrera et al. 2006). Among them, seven identified proteins were found significantly altered in almost all the AML blast cells analyzed in relation to normal mononuclear blood cells: alpha-enolase, RhoGDI2, annexin A10, catalase, peroxiredoxin 2, tromomyosin 3, and lipocortin 1 (annexin 1). These differentially expressed proteins are known to play important roles in cellular functions such as glycolysis, tumor suppression, apoptosis, angiogenesis, and metastasis, and they might contribute to the adverse evolution of the disease. Using similar proteomic techniques, other proteins have been identified that are expressed differentially in AML: alpha-2-HS-glycoprotein, complement-associated protein SP-40, RBP4 gene product, and lipoprotein C-III are downregulated, whereas immunoglobulin heavy-chain variant, proteasome 26S ATPase subunit 1, and haptoglobin-1 are upregulated. Proteomic analysis has identified novel proteins that may either help to determine a differential prognosis or be used as biomarkers for disease outcome, thus providing potential new targets for rational pathogenesis-based therapies of AML.

5.5.8.6 Biomarkers of Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL), which is usually diagnosed at early stages, has an extremely variable individual prognosis, as some patients remain stable for years, whereas others rapidly develop aggressive forms of the disease. Biomarkers provide useful information for prognosis. These include cytogenetic abnormalities diagnosed by FISH, mutational status of the immunoglobulin genes (IgVH), and expression of zeta-associated protein 70 (ZAP-70). Some notable findings are (Véronèse et al. 2008; Gribben 2008) as follows:

- The detection of del 17p or del 11q is associated with poor risk, while del 13q as a sole abnormality is associated with low risk of disease.
- Nearly half of CLL cases have somatic hypermutation in the variable regions of the genes, and this has prognostic significance.
- Some studies have suggested that ZAP70 status is more useful as a predictor of time to progression than mutation status, but this remains controversial.
- Most of the modern prognostic markers were validated by retrospective analysis and have now been applied to prospective randomized clinical trials. These studies suggest that the same molecular biomarkers that identify patients with more aggressive disease also have impact on outcome after treatment.

Several CLL risk factor biomarkers (immunoglobulin heavy-chain variable region mutational status, CD38 and ZAP-70 expression, CCL3 plasma levels) are related to B-cell receptor (BCR) function and signaling, a key factor in CLL pathogenesis (Burger 2012). With the availability of inhibitors of BCR-associated kinases (Btk-, Syk-, and PI3-kinase inhibitors) in CLL and other B-cell malignancies, BCR-related risk factors may help with identification of patients who are likely to respond and/or for response assessment (i.e., CCL3 plasma levels).

A study has shown that circulating miRNAs can be sensitive biomarkers for CLL, because certain extracellular miRNAs are present in CLL patient plasma at levels significantly different from healthy controls and from patients affected by other hematological malignancies. The levels of several of these circulating miRNAs also displayed significant differences between ZAP-70+ and ZAP-70− CLL (Moussay et al. 2011). The authors also determined that the level of circulating miR-20a correlates reliably with diagnosis-to-treatment time. Network analysis of their data suggests a regulatory network associated with bcl2 and ZAP-70 expression in CLL suggesting the possibility of using the levels of specific miRNAs in plasma to detect CLL as well as to determine the ZAP-70 status.

5.5.8.7 Biomarkers of Chronic Myeloid Leukemia

CML is a clonal multistep myeloproliferative disease that is initially produced and ultimately sustained by a rare subpopulation of BCR–ABL+ cells with multi-lineage stem cell properties. These BCR–ABL+ CML stem cells are phenotypically similar to normal hematopoietic stem cells which are also maintained throughout the course of the disease at varying levels in different patients. Defining the unique properties of the leukemic stem cells that produce the chronic phase of CML has therefore had to rely heavily on access to samples from rare patients in which the stem cell compartment is dominated by leukemic elements. A study has reviewed past and ongoing approaches using such samples to identify biologically and clinically relevant biomarkers of BCR–ABL+ stem cells that explain their unusual biology and that may help to design, or at least predict, improved treatment responses in CML patients (Jiang et al. 2008).

Genome-wide expression profiling of miRNAs can be carried out in CML by using a microarray containing hundreds of human precursor and mature miRNA oligonucleotide probes. miRNA expression profiles can be used to distinguish normal B cells from malignant B cells in patients with CLL. A study has evaluated the miRNA expression profiles of samples of CLL cells and the genomic sequence of 42 miRNA genes to identify abnormalities (Calin et al. 2005). A unique miRNA signature is associated with prognostic factors and disease progression in CLL. The study also showed that mutations in miRNA transcripts are common and may have functional importance.

Expression of the gene encoding HAAH in leukocytes can be measured in patients with CML to identify those patients unlikely to respond to therapeutic treatment with imatinib mesylate (Gleevec®). Although the molecular hallmark of CML

is a mutation in a gene known as BCR–ABL, mutations of the ABL portion of the gene do not reliably predict response to Gleevec® therapy. Expression of the gene encoding HAAH significantly decreases when leukocytes from patients with CML are cultured in the presence of imatinib. This decrease in HAAH gene expression level correlates with drug response. Patients not responding to imatinib treatment do not show a decrease in HAAH expression in the assay. An assay for quantitative measurement of HAAH in human serum is also available.

5.5.8.8 Biomarkers of Drug Resistance in Leukemia

It is unclear why some patients develop resistance to imatinib mesylate or other anticancer agents and what can be done to prevent or delay the onset of resistance. With regard to imatinib, resistance has been associated with several mechanisms including (1) amplification or mutations of the BCR–ABL fusion gene, (2) inactivation by binding to α-1 acid glycoprotein, and (3) increased usage of signal transduction pathways that are BCR–ABL independent. However, these pathways remain undefined.

5.5.8.9 Biomarkers of Myelodysplastic Syndromes

Myelodysplastic syndromes (MDS) are among the most frequent hematological malignancies. Patients have a short survival and often progress to AML. The diagnosis of MDS can be difficult; there is a paucity of molecular markers, and the pathophysiology is largely unknown. A multicenter study was conducted to investigate whether serum proteome profiling may serve as a noninvasive platform to discover novel molecular biomarkers for MDS (Aivado et al. 2007). Peptide mass fingerprinting and quadrupole TOF MS identified two differential proteins: CXC chemokine ligands 4 (CXCL4) and 7 (CXCL7), both of which had significantly decreased serum levels in MDS, as confirmed with independent antibody assays revealed that these two proteins have decreased serum levels in advanced MDS, suggesting the possibility of a concerted disturbance of transcription or translation of these chemokines in advanced MDS.

5.5.9 Lymphoma Biomarkers

Lymphoma begins in the lymphatic cells of the immune system and presents as a solid tumor of lymphoid cells. There are three large groups: the B-cell (including lymphocytic leukemia described in previous section 5.5.8.6), T-cell, and natural killer (NK) cell tumors. Novel biomarkers for categorization or risk stratification in patients with diffuse large B-cell lymphoma are being developed and validated. Hodgkin lymphoma is a tumor of abnormal lymphocytes of mature B-cell lineage.

Several HDAC inhibitors are in development, many of which have been shown preclinically to have potent antitumor activity. Clinical trials using these agents are now underway, with Vorinostat (suberoylanilide hydroxamic acid) having been approved by the FDA for treating cutaneous T-cell lymphoma (CTCL) in patients with progressive, persistent, or recurrent disease. HR23B is a candidate cancer biomarker identified in a genome-wide loss-of-function screen which influences sensitivity to HDAC inhibitors. A study has shown that HR23B governs the sensitivity of CTCL cells to HDAC inhibitors (Khan et al. 2010). Furthermore, proteasome activity is deregulated in HDAC inhibitor-treated CTCL cells through a mechanism dependent upon HR23B, and HDAC inhibitors sensitize CTCL cells to the effects of proteasome inhibitors. The predictive power of HR23B for clinical response to HDAC inhibitors was investigated through an analysis of a unique collection of CTCL biopsies taken from a phase II clinical trial, where there was a frequent coincidence between HR23B expression and clinical response to HDAC inhibitor, supporting the biomarker-based approach to personalized treatment of cancer.

5.5.10 Liver Cancer Biomarkers

Liver cancer is the fifth most common cancer in the world and one of the deadliest cancers since it is rarely diagnosed until late in its development. The lack of reliable screening tests for liver cancer contributes to its high mortality rate since tumors seldom cause symptoms until the later stages when treatment options become limited and the prognosis is poorer. Death usually occurs not long after diagnosis.

It is important to detect HCC and the recurrence at its earlier period. Serum tumor biomarkers for detecting HCC can be divided into four categories: oncofetal antigens and glycoprotein antigens, enzymes and isoenzymes, genes, and cytokines (Zhou et al. 2006). Serum AFP is the most widely used tumor biomarker in detecting patients with HCC. Some tumor biomarkers, such as human cervical cancer oncogene and hTERT mRNA, are considered to be more accurate than AFP. Other tumor biomarkers, such as glypican-3, gamma-glutamyl transferase II, alpha-L-fucosidase, transforming growth factor-beta1 (TGF-β1), and tumor-specific growth factor, are available as supplements to AFP in the detection of HCC. AFP mRNA has been shown to correlate with the metastasis and recurrence of HCC, and it may be the most useful biomarker for prognosis.

Des-gamma-carboxy prothrombin (DCP) has been reported to be more sensitive and specific in diagnosing HCC when compared with AFP. One study indicates that DCP has a better diagnostic value than AFP in differentiating HCC from nonmalignant chronic liver disease (Wang et al. 2005). DCP has not only a stronger correlation with HCC than AFP in tumor size but also more effectiveness than AFP in detecting small size of HCC. Biomarkers, such as gamma-glutamyl transferase mRNA, vascular endothelial growth factor, and interleukin-8, could also be

used as available prognostic indicators, and the simultaneous determination of AFP and these markers may detect the recurrence of HCC at its earlier period. Serum RCP levels are significantly elevated in HCC also and could potentially serve as a marker for HCC detection under conditions where breast cancer is ruled out (Rao et al. 2006).

A chromatin-remodeling enzyme, ALC1 (Amplified in Liver Cancer 1), also known as CHD1L, which interacts with poly(ADP-ribose) and catalyzes PARP1-stimulated nucleosome sliding, has been identified (Ahel et al. 2009). It is defined as a DNA damage-response protein, whose role in this process is sustained by its association with known DNA repair factors and its rapid poly(ADP-ribose)-dependent recruitment to DNA damage sites. Depletion or overexpression of ALC1 results in sensitivity to DNA-damaging agents. Collectively, these results provide new insights into the mechanisms by which poly(ADP-ribose) regulates DNA repair. ALC1 is found in excessive amounts in half of liver cancers and can be used as a biomarker to pinpoint when liver cells start to become cancerous.

Several studies have shown that the blood levels of Golgi Protein-73 (GP73), a type II Golgi-localized integral membrane protein, are consistently higher in patients with primary liver cancer than in healthy individuals. In addition, levels are not significantly higher in patients with diseases other than liver disease. GP73 is a biomarker of HCC (Fimmel and Wright 2009). It is being tested in clinical trials and several medical diagnostic companies are developing automated serum tests for GP73 that could be performed in routine hospital laboratories. As a new diagnostic biomarker of PHC, GP73 protein in serum was highly sensitive and specific. Another study has shown that the combined detection of GP73 and AFP in serum effectively improves the diagnosis of HCC (Shi et al. 2011).

5.5.10.1 Metabonomic Profiles Discriminate HCC from Liver Cirrhosis

Biomarkers that discriminate HCC from LC are important but are limited. An ultra-performance LC–MS (UPLC–MS)-based metabonomic approach was used to characterize serum profiles from HCC, liver cirrhosis, and healthy subjects; the accuracy of UPLC–MS profiles and AFP levels was compared for their use in HCC diagnosis (Wang et al. 2012). By multivariate data and receiver operating characteristic curves analysis, metabolic profiles were capable of discriminating not only patients from the controls but also HCC from LC with 100 % sensitivity and specificity. Thirteen potential biomarkers were identified and suggested that there were significant disturbances of key metabolic pathways, such as organic acids, phospholipids, fatty acids, bile acids, and gut flora metabolism, in HCC patients. Canavaninosuccinate was first identified as a metabolite that exhibited a significant decrease in LC and an increase in HCC. In addition, glycochenodeoxycholic acid was suggested to be an important indicator for HCC diagnosis and disease prognosis. UPLC–MS signatures, alone or in combination with AFP levels, could be an efficient and convenient tool for early diagnosis and screening of HCC in high-risk populations.

5.5.11 Lung Cancer Biomarkers

Lung cancer is the leading cause of cancer-related death in Western nations. More than 300 million people die of this disease annually. In the USA alone, 170,000 new cases of lung cancer are reported each year. Lung cancer is broadly divided into two types. Small-cell lung cancer (SCLC) accounts for approximately 80 % of the all lung cancers and has a potential for cure by surgical resection. Non-small-cell lung cancer (NSCLC), an epithelial tumor, comprises about 20 % of all lung cancers and has a highly aggressive clinical course with tendency for early widespread metastases. Most of these are NSCLC and the overall prognosis once diagnosed is dismal. The only reasonable chance of cure is surgical resection for early-stage tumors. However, most patients with early lung cancer are asymptomatic. Symptoms usually develop after the tumors become invasive or disseminated and curative resection is infeasible. Consequently, researchers have been working to find novel noninvasive or semi-invasive methods of identifying individuals who harbor progressive precancerous lesions. If detected early, these lesions might be treated with a chemopreventive agent to impede progress to invasive carcinoma.

Currently sputum cytology is considered to be the gold standard to assess the presence of malignant cells. Molecular biomarkers need to be validated before they are used in early clinical trials. Biomarkers for lung cancer are primarily involved in one of three major pathways: cell cycle regulation, apoptosis, and angiogenesis. Although no single biomarker has yet been shown to be perfect in predicting patient outcome, a profile based on the best of these biomarkers may prove useful in directing patient therapy. Various biomarkers for lung cancer are listed in Table 5.8.

5.5.11.1 Autoantibodies as Biomarkers in Lung Cancer

Immune response manifested by annexin I and II autoantibodies occurs commonly in lung cancer and is associated with high circulating levels of IL-6—an inflammatory cytokine. A proteomic approach using 2D PAGE, followed by Western blot analysis in which individual sera were tested for primary antibodies, has led to the discovery of antiannexins I and/or II in sera from patients with lung cancer. Biomarkers have been detected in 90 % of cases of lung adenocarcinoma. The CARET (Carotene and Retinol Efficacy Trial) feasibility study showed that antiannexin antibodies could be detected in serum samples collected a year prior to clinical diagnosis of lung cancer.

Researchers at the University of Kentucky have described a fluorescent protein microarray to identify and measure multiple NSCLC-associated antibodies and showed how simultaneous measurements can be combined into a single diagnostic assay. Measurements of the five most predictive phage proteins were combined in a logistic regression model that achieved 90 % sensitivity and 95 % specificity in

Table 5.8 Biomarkers of lung cancer

Detection of cancer cells in sputum
Biomarkers in blood
Direct detection of circulating tumor cells in the blood
Tumor-derived DNA and RNA markers in blood
Biomarkers in exhaled breath
miRNA biomarkers in lung cancer
Protein biomarkers
Antibodies: annexins I and II
Apolipoprotein C-I
Haptoglobin alpha-1 chain
S100A4
Serum protein biomarkers
Serum tNOX (tumor-associated NOX)
Gene expression profiling for biomarkers of lung cancer
Alterations of chromosomes: 3p, 5q, 9p
Genes: Rb, C-myc, C-mos, hTERT, K-ras, BRCA1, caveolin-1 (CAV1), and caveolin-2 (CAV2)
Proteins: p16, p53, hnRNP A2/B1, MCM2, EGFR, erbB-2, erbB-3, erbB-4, cyclophilin A (CyPA), CYPHRA21-1
Airway epithelial cell gene expression
miRNA expression in bronchial epithelium of smokers
Biomarkers closely associated with neuroendocrine differentiation in NSCLC
Progastrin-releasing peptide (ProGRP)
Neuron-specific enolase (NSE)
Chromogranin A (CGA)
Biomarkers of inflammation
Cytokines: IL-6
C-reactive protein (CRP)
Methylation biomarkers
Nucleosomes in serum
Vascular endothelial cell growth factor

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prediction of patient samples, whereas leave-one-out statistical analysis achieved 88.9 % diagnostic accuracy among all 81 samples. In testing this marker set with samples from the Mayo Clinic Lung Screening Trial, the authors correctly predicted six of six prevalence cancers, 32 of 40 cancers from samples drawn 1–5 years before radiographic detection on incidence screening, and 49 of 56 risk-matched controls (Zhong et al. 2006). These data indicate that antibody profiling is a promising approach that could achieve high diagnostic accuracy for NSCLC. These biomarkers are being developed into a test for lung cancer. Panels of autoantibody serum assays are also being developed and are unique in that they are designed to detect the first changes in proteins shown when cancer is developing. The assays have been optimized to display the proper epitopes in the proper orientation to bind to autologous antibodies.

5.5.11.2 Biomarkers Associated with Neuroendocrine Differentiation in NSCLC

Progastrin-releasing peptide (ProGRP), along with chromogranin A (CGA) and neuron-specific enolase (NSE), belongs to a group of immunohistochemical tissue biomarkers closely associated with neuroendocrine differentiation in NSCLC. Serum levels of these biomarkers can also be measured and are used in predicting response to chemotherapy and survival of patients with unresectable NSCLC. In a study of NSCLC patients on chemotherapy, serum CGA and ProGRP were reported to provide important information related to the prognosis for NSCLC patients before chemotherapy (Nisman et al. 2006). While a high CGA before treatment was found as an unfavorable prognostic determinant, a high ProGRP conferred a survival advantage. It was concluded that the combined use of serum CGA, ProGRP, and NSE may supply additional information to prognosis. In another study, tumor marker serum levels were related to histological type and tumor extension, with ProGRP being the most sensitive marker in SCLC, CEA in adenocarcinomas, and CYFRA 21-1 in squamous tumors of the lung (Molina et al. 2005). The most sensitive combinations of tumor markers were ProGRP and NSE in SCLC (88 %), and CEA plus CYFRA 21-1 in NSCLC (82 %). Thus, ProGRP is the tumor biomarker of choice in SCLC, and NSE is a complementary tumor biomarker for this histological type.

5.5.11.3 Biomarkers of Chronic Inflammation in Lung Cancer

Chronic inflammation has been implicated in the development of airway dysplasia and lung cancer. C-reactive protein (CRP), a biomarker for inflammation in the blood, can help to identify individuals whose abnormal precancerous lesions will advance closer to invasive lung cancer. Plasma CRP, in concert with lung function and pack-years of smoking, appears to have excellent predictive powers in identifying participants with bronchial dysplastic lesions whose lesions progress to more advanced stages of dysplasia (Sin et al. 2006). The odds of developing progressive disease were 9.6-fold higher in the group that had CRP greater than 0.5 mg/L compared with the group less than this threshold.

5.5.11.4 Biomarkers for Predicting Sensitivity to Chemotherapy in Lung Cancer

Platinum-based chemotherapy is the standard treatment in advanced NSCLC and as adjuvant treatment in a substantial subgroup of patients with stage II–IIIA. Therefore, there is an interest in biomarkers for predicting sensitivity to platinum compounds. The expression of genes involved in DNA repair pathways, particularly genes involved in the nucleotide excision repair, has an important role in predicting response to platinum-based chemotherapy. Therefore, patients with low DNA repair capacity should have a favorable response to gemcitabine plus carboplatin

chemotherapy, whereas they could be resistant to docetaxel. However, cisplatin can induce ERCC1 (excision repair cross-complementing 1) mRNA levels, which might be a principal reason for short-lived response to platinum agents. Although several trials evaluated the level of expression of ERCC1, no consensus was reached regarding a method for evaluation. A study has used the 8F1 antibody to measure the level of expression of ERCC1 by IHC analysis in a validation set of samples obtained from patients in two independent phase III trials (Friboulet et al. 2013). Unique functional ERCC1 isoform was not specifically detected; therefore, its usefulness in guiding therapeutic decision-making is limited. BRCA1 complexes are also central to DNA repair response, and the influence of RAP80 expression in conjunction with BRCA1 expression is now being investigated in phase III randomized clinical trials of customized chemotherapy.

In patients with inoperable SCLC, the efficacy of chemotherapy can be predicted early in the course of therapy by baseline values of serum nucleosomes as independent parameters. In prospectively collected sera of patients with recurrent NSCLC receiving second-line chemotherapy, the courses of nucleosomes, cytokeratin-19 fragments (CYFRA 21-1), CEA, NSE, and progastrin-releasing peptide were investigated and correlated with therapy response (Holdenrieder et al. 2009). At high specificity for detection of progressive disease, most sensitive biomarkers were identified and included in a combination model. High levels and insufficient decreases of nucleosomes and CYFRA 21-1 during the first cycle of therapy indicated poor outcome. Combination of nucleosome concentrations and CYFRA 21-1 enabled the early detection of progressive disease. Nucleosomes and CYFRA 21-1 were shown to be valuable for the individual management of patients with recurrent NSCLC.

Several other biomarkers of response of lung cancer to chemotherapy have been identified. There is need for clinical trials to prospectively validate the benefits of customizing chemotherapy for translation into an improvement in outcome of NSCLC patients.

5.5.11.5 Biomarkers for Prediction of Sensitivity to EGFR Inhibitors

EGFR inhibitors have shown promising results in patients with advanced NSCLC who previously have failed on chemotherapy. Objective response is achieved in 10–28 % of the patients, and in ~30 % the disease becomes stable (Hirsch and Witta 2005). A major problem is how to select the patients, who will benefit from treatment and who will not. The predictive role of EGFR protein expression assessed by IHC is still debated. Specific EGFR gene mutations have been identified associated with response to gefitinib (Iressa®), but seem not to be associated with stable disease. Other biomarker studies are described, which are associated with sensitivity to EGFR inhibitors. Increased EGFR gene copy number based on FISH analysis is demonstrated to be a good predictive marker for response, stable disease, time to progression, and survival. However, EGFR mutation is a better predictor of clinical outcome in gefitinib-treated patients than the EGFR gene copy number (Endo et al. 2006). EGFR/FISH seems today to be the best predictive marker for clinical benefit from

EGFR inhibitors in NSCLC. Prospective large-scale clinical studies must identify the most optimal paradigm for selection of patients.

Previously, tumor biopsies have been used for EGFR genotyping in NSCLC as it has been difficult to detect the low levels of specific mutations shed from the tumor into the blood against the high background of normal DNA. Testing DNA isolated from blood, rather than tumor tissue, would be better for predicting responses to gefitinib, erlotinib (Tarceva), and other cancer therapies. If EGFR mutations can be observed in serum DNA, this could serve as a noninvasive source of information on the genotype of the original tumor cells as compared to direct sampling of the tumor and could influence treatment and the ability to predict patient response to gefitinib. In one study, serum genomic DNA was obtained from Japanese patients with NSCLC before first-line gefitinib monotherapy (Kimura et al. 2006). Scorpion Amplified Refractory Mutation System technology (DxS Ltd.) was used to detect EGFR mutations. In pairs of tumor and serum samples obtained from patients, the EGFR mutation status in the tumors was consistent with those in the serum of over 72 % of the paired samples. The DxS test kit detected mutations that were missed by direct sequencing techniques. These results suggest that patients with EGFR mutations seem to have better outcomes with gefitinib (Iressa) treatment, in terms of progression-free survival, overall survival, and response, than those patients without EGFR mutations.

A phase I, dose-escalation, combination drug study of subjects with NSCLC receiving celecoxib and erlotinib (Tarceva) identified proteins that may serve as biomarkers to guide advanced lung cancer treatment (Krysan et al. 2008). All of these advanced lung cancer patients had previously received conventional treatments without success. About half of the patients had positive outcomes on the therapy defined as tumors that did not grow or that decreased in size by more than 30 %. Use of ELISA to analyze patients' serum protein levels at various time points over 2 months revealed an intriguing link between treatment response and the levels of four proteins: soluble E-cadherin, a MMP, a tissue inhibitor of MMP, and CCL15. These proteins act downstream of COX-2, an enzyme causing inflammation that can hinder cancer treatment by vascularizing tumors and making them more resilient. COX-2, which is overexpressed in 80–85 % of lung cancers, also seems to hinder the effect of drugs such as erlotinib, which target EGFR on tumor cell surfaces. Combination therapy with the COX-2 inhibitor celecoxib is intended to restore tumor cell sensitivity and enhance erlotinib's efficacy, since only 15 % of patients respond to erlotinib alone. In this study, the patients who showed positive outcomes after 8 weeks also had lower sEC, TIMP-1, and CCL15 serum levels, while patients with low MMP-9 levels before treatment showed the best treatment results. The latter result indicates the usefulness of MMP-9 as a treatment biomarker for determining the appropriateness of the new combination drug treatment. A larger, phase II, multicenter study will verify their preliminary association between tumor size and protein levels during combination drug treatment.

In 2008, the NIH started a 4-year phase III study, Marker Validation for Erlotinib in Lung Cancer (MARVEL), to validate if choosing patients with NSCLC for erlotinib based on the presence or absence of EGFR activation has a meaningful benefit over the standard chemotherapy the patients received.

5.5.11.6 CTCs as Biomarkers of Lung Cancer

In lung cancer, the biological assessment of CTCs therapeutic target biomarkers, such as mutations of EGFR, could be a valid alternative to its determination in tumor samples. EGFR mutation analysis in CTC was shown to be concordant with data from tumoral samples (Maheswaran and Haber 2010). CTCs characteristics in lung cancer could help to stratify the patients and possibly drive future therapeutic strategies. Numbers of CTCs correlate with prognosis in both early and advanced lung cancers. The availability of this noninvasive virtual biopsy could solve the problem of the increasing need of lung cancer biological samples for molecular studies. This would help avoid the use of invasive procedures in order to obtain cytological specimens or small biopsies (Franco et al. 2012).

5.5.11.7 Gene Expression Profiling for Biomarkers of Lung Cancer

The most common genetic changes associated with lung cancer involve abnormalities of the genes and the proteins expressed by them, which regulate the cell cycle. Molecular networking of P53 and P16 tumor suppressor genes and K-ras oncogene exerts a crucial impact on cell cycle regulation and appears to be of major clinical significance for lung cancer evaluation. P53, P16, and K-ras evaluations have been used in lung cancer with particular focus on biological and clinical implications, as well as on new molecular approaches to the study of these genes.

Sixteen genes that correlated with survival among patients with NSCLC were identified by analyzing microarray data and risk scores (DUSP6, MMD, STAT1, ERBB3, and LCK) were selected for RT-PCR and decision-tree analysis (Chen et al. 2007). The five-gene signature is closely associated with relapse-free and overall survival among patients with NSCLC. BRCA1 and xeroderma pigmentosum group G (XPG) are independent prognostic factors for both median survival and disease-free survival. High BRCA1 mRNA expression confers poor prognosis in early NSCLC, and the combination of high BRCA1 and low XPG expression still further increases the risk of shorter survival (Bartolucci et al. 2009). These findings can help optimize the customization of adjuvant chemotherapy.

A subset of 11 genes has been identified as a prognostic gene expression signature and validated in multiple independent NSCLC microarray datasets (Navab et al. 2011). Functional annotation using protein–protein interaction analyses of these and published cancer gene expression changes revealed prominent involvement of the focal adhesion and MAPK signaling pathways. Fourteen of the 46 genes were also differentially expressed in LCM primary tumor stroma compared with the matched normal lung. Six of these 14 genes could be induced by TGF-β1 in normal fibroblasts. These results establish the prognostic impact of changes in gene expression of carcinoma-associated fibroblasts in NSCLC patients.

Because cigarette smoke injures the airway, efforts have been made to determine if gene expression in histologically normal large-airway epithelial cells obtained at bronchoscopy from smokers with suspicion of lung cancer could be used as a lung

cancer biomarker. Using a training set and gene expression profiles from Affymetrix HG-U133A microarrays, an 80-gene biomarker was identified that distinguishes smokers with and without lung cancer (Spira et al. 2007). This biomarker had ~90 % sensitivity for stage 1 cancer across all subjects. Combining cytopathology of lower airway cells obtained at bronchoscopy with the biomarker yielded 95 % sensitivity and a 95 % negative predictive value. These findings indicate that gene expression in cytologically normal large-airway epithelial cells can serve as a lung cancer biomarker, potentially owing to a cancer-specific airway-wide response to cigarette smoke.

5.5.11.8 Methylation Biomarkers of Lung Cancer

Methylation profiling, a DNA analysis technique, can identify molecular biomarkers of early lung cancer. Biomarkers of lung cancer have also been identified by differential methylation hybridization technology and have been extensively validated on tissue samples before being tested on blood plasma. A large clinical trial has confirmed that a two-biomarker panel correctly identified two-thirds of all lung cancers in blood plasma at a false-positive rate of 12 % (88 % specificity). Most of the blood samples used in the study were obtained from patients with early-stage I and II cancer. Sensitivity in stage II lung cancer patients reached 73 %. Patients with early stage cancer are significantly underdiagnosed in the current diagnostic practice for lung cancer but could benefit most from early therapeutic intervention.

Despite optimal and early surgical treatment of NSCLC, many patients die of recurrence. This led to investigation of the association between gene methylation and recurrence of the tumor. In a multivariate model, the following were associated with tumor recurrence, independently of NSCLC stage, age, sex, race, smoking history, and histological characteristics of the tumor (Brock et al. 2008):

1. Promoter methylation of the cyclin-dependent kinase inhibitor 2A gene p16
2. H-cadherin gene CDH13
3. Ras association domain family 1 gene RASSF1A
4. Adenomatous polyposis coli gene APC in tumors and in histologically tumor-negative lymph nodes

It was concluded that methylation of the promoter region of the four genes in patients with stage I NSCLC treated with curative intent by means of surgery is associated with early recurrence.

5.5.11.9 miRNA Biomarkers in Lung Cancer

Let-7, a natural and separately transcribed miRNA, maps to a chromosomal region associated with lung cancer as a regulator of Ras expression. Let-7 expression is lower in lung tumors than in normal lung tissue, while RAS protein is significantly higher in lung tumors, providing a possible mechanism for let-7 in cancer. The let-7 miRNA regulates Ras by binding to the message for Ras and likely inhibits

translation of the Ras protein rather than reversion of a mutated Ras to normal. The LCS6 variant allele in a K-ras miRANA complementary site is significantly associated with increased risk for NSCLC among moderate smokers and links let-7 miRNAs to lung cancer susceptibility (Chin et al. 2008). These findings open up the possibility that gene therapy with let-7 may alleviate or slow down lung cancer.

Studies of whole-genome miRNA and mRNA expression in bronchial airway epithelial brushings obtained at bronchoscopy from smokers have revealed 28 miRNAs to be differentially expressed in the majority of smokers. Modulation of the expression of one of these miRNAs, mir-218, was sufficient to alter the expression of a subset of the mRNAs that are both predicted targets of this miRNA and are altered by smoking in vivo. These studies suggest that smoking-dependent changes in miRNA expression levels mediate some of the gene expression changes in airway epithelium induced by smoking and that miRNAs, therefore, play a role in the host response to environmental exposures and may contribute to the pathogenesis of smoking-related lung cancer. It is hoped that miRNA profiles obtained from these cells may serve as relatively noninvasive biomarkers for smoking-related lung diseases.

A study has explored miRNA expression profiles of lung tumors, normal lung tissues, and plasma samples from cases with variable prognosis identified in a completed spiral-CT screening trial with extensive follow-up (Boeri et al. 2011). miRNA expression patterns significantly distinguished (a) tumors from normal lung tissues, (b) tumor histology and growth rate, (c) clinical outcome, and (d) year of lung cancer CT detection. Thus, miRNAs play a role in lung tissues and plasma as molecular predictors of lung cancer development and aggressiveness and have theoretical and clinical implication for lung cancer management.

5.5.11.10 Proteomic Biomarkers in Exhaled Breath Condensate

Exhaled breath condensate (EBC) collection is a simple and noninvasive technique, which enables sampling of lower respiratory tract fluid. EBC may be applied to the detection of lung cancer where it could be a tool in early diagnosis. Proteomic analysis of alveolar fluid obtained in the form of EBC can be valuable for detecting and effectively diagnosing lung cancer. A review of proteomic EBC analysis along with discussion of benefits, pitfalls, and possible future development of this approach has been published (Eberini et al. 2008). Advances in proteomic technologies are expected to validate EBC proteins as biomarkers of lung cancer that may enable early detection and improve the outcome of treatment.

5.5.11.11 Serum Protein Biomarkers of Lung Cancer

There is an enormous unmet medical need related to the diagnosis of lung cancer in the earliest stages when it is most treatable, but no approved blood test for lung cancer is yet available. Serum biomarkers that could aid clinicians in making case management decisions about lung cancer would be extremely useful. Two

proteomic platforms and literature search have enabled selection of candidate serum biomarkers for the diagnosis of lung cancer (Patz et al. 2007). Classification and Regression Tree (CART) analysis was used to select a panel of four serum protein biomarkers for prediction of lung cancer in patients: (1) CEA, (2) retinol binding protein, (3) α1-antitrypsin, and (4) squamous cell carcinoma antigen. These were collectively found to correctly classify the majority of lung cancer and control patients in the training set (sensitivity 89.3 % and specificity 84.7 %). These biomarkers also accurately classified patients in the independent validation set (sensitivity 77.8 % and specificity 75.4 %). Approximately 90 % of patients who fell into any one of three groupings in the CART analysis had lung cancer. Thus, the panel of four serum proteins is valuable in the diagnosis of lung cancer. The data may be useful for treating patients with an indeterminate pulmonary lesion and potentially in predicting individuals at high risk for lung cancer. Serum protein assays for detection of biomarkers could serve as a useful complement to imaging studies such as CT scan to differentiate cancers from benign nodules.

An efficient strategy, consisting of SELDI-TOF MS analysis, HPLC purification, MALDI-TOF MS trace, and LC–MS/MS identification, is useful for the detection of protein biomarkers. Apolipoprotein C-I, haptoglobin alpha-1 chain, and S100A4 have been identified as potential proteomic biomarkers of NSCLC, but further studies with larger sample sizes will be needed to validify these (Yang et al. 2009b).

5.5.11.12 tNOX as Biomarker of Lung Cancer

tNOX (tumor-associated NOX) is a member of a family of proteins that are involved in cell growth. Normal cells express the NOX enzyme only when they are dividing in response to growth hormone signals. In contrast, cancer cells have gained the ability to express NOX activity at all times. This overactive form of NOX, known as tNOX, is vital for the growth of cancer cells, because drugs that inhibit tNOX activity also block tumor cell growth in culture.

NOX Technologies Inc., in collaboration with scientists at Purdue University (West Lafayette, Indiana), is developing serum tNOX test as a screening tool for the early detection of lung cancer. Those who test positive would then be followed up with a medical examination and further tests, ostensibly including high-resolution CT. This test is structured with the antibody for lung cancer in one form or another and is a specific diagnosis that also distinguishes between non-small-cell and small-cell lung cancer. Preliminary tests in subjects with lung cancer are encouraging for accuracy of this test. Tests based on tNOX are also in development for other cancers.

5.5.11.13 Tumor-Derived DNA and RNA Biomarkers in Blood

PCR enables the detection and quantification of extremely small amounts of tumor-derived nucleic acids. This has led to an increased knowledge of the molecular pathogenesis of lung cancer and a basis for the use of DNA and RNA biomarkers in

blood for early cancer detection, diagnostics, and follow-up. Common genetic alterations in lung carcinogenesis are already well known. Several clinical studies have evaluated the role of DNA and RNA aberrations in the blood of lung cancer patients and overall plasma/serum abnormalities were found in 43 % of patients with lung cancer and 0.8 % of healthy controls (Bremnes et al. 2005). The analysis of circulating DNA or RNA in plasma is a promising noninvasive diagnostic tool, requiring only a limited blood sample. Its wide applicability and potential importance will possibly lead to increasing clinical impact in the near future. However, large prospective clinical studies are needed to validate and standardize any tests for DNA or RNA alteration in plasma or serum of high-risk individuals or patients with established lung cancer.

5.5.11.14 Volatile Organic Compounds in the Exhaled Breath

The pattern of VOCs in the exhaled breath of lung cancer patients may be unique. Novel sensor systems that detect patterns of volatiles have been developed. One of these sensor systems, a colorimetric sensor array, has 36 spots composed of different chemically sensitive compounds impregnated on a disposable cartridge (Mazzone et al. 2007). The colors of these spots change based on the chemicals they come in contact with. The color changes that occur for each individual are converted into a numerical vector. The vectors were analyzed statistically, using a random forests technique, to determine if lung cancer could be predicted from the sensor responses. A prediction model was developed using observations from 70 % of the subjects with various lung diseases including cancer. This model was able to predict the presence of lung cancer in the remaining 30 % of the subjects with a sensitivity of 73.3 % and specificity of 72.4 %. Thus, the unique chemical signature of the breath of lung cancer patients can be detected with moderate accuracy by a colorimetric sensor array.

5.5.12 Malignant Pleural Mesothelioma

Malignant pleural mesothelioma (MPM) is a highly aggressive neoplasm of lung pleura with poor prognosis. Exposure to asbestos fibers is the primary cause of MPM with as many as 80 % of the patients having been exposed to asbestos. Recent age standardization rates for mesothelioma in men in Australia are estimated to be 6 per 100,000. Although MPM remains a relatively uncommon malignancy, it continues to represent an important cause of mortality in numerous areas worldwide, e.g., England, Wales, continental Europe, and Australia.

MPM remains difficult to detect early and treat effectively. Novel proteomic technologies can be utilized to discover changes in expression of pleural proteins that might have diagnostic value. SELDI-TOF and MS can be used to detect protein

profiles in pleural effusions that could identify MPM. Mesomark™ (Fujirebio Diagnostics Inc.) is an ELISA for the quantitative measurement of soluble mesothelin-related peptides (SMRP) in human serum that are related to the mesothelin/megakaryocyte potentiating factor (MPF) family of proteins and recognized by the MAb OV569. The reactivity of OV569 is low for normal human tissues except for the mesothelium. In a study on pleural effusions from patients with confirmed MPM and from patients with effusions due to other causes, various commercially available immunoassays were used to detect human epididymis protein 4 (HE4), osteopontin (OPN), SMRP, and the cytokeratin-19 fragment (CYFRA 21-1), and peak intensity data obtained by SELDI-TOF were subjected to classification algorithms in order to identify potential classifier peaks (Hegmans et al. 2009). A protein peak at *m/z* 6614 was characterized as apolipoprotein (Apo) CI. In this setting, the sensitivity and specificity of the potential biomarker, Apo CI, was 76 % and 69 %, respectively, thereby outperforming OPN, HE4, and CYFRA 21-1. This study validates the use of SMRP (as measured by Mesomark™) as a diagnostic biomarker for pleural mesothelioma and furthermore suggests that Apo CI levels could be used in the future to discriminate MPM from other causes of pleural exudate.

5.5.13 Melanoma Biomarkers

Cutaneous malignant melanoma remains the leading cause of skin cancer death in industrialized countries. Melanoma is diagnosed in more than 50,000 new patients in the USA annually. Melanoma progression is well defined in its clinical and histopathological aspects (Breslow's index, tumor size, ulceration, or vascular invasion), which also give hints to prognosis of the patient. Use of molecular biomarkers should therefore give additional information which cannot be determined by routine histopathology. There is a critical unmet need for new predictive and prognostic biomarkers for melanoma, particularly ones that can identify those tumors that are likely to result in progression (metastasis) and death. A classification of biomarkers of melanoma is shown in Table 5.9.

Several molecules influencing invasiveness and metastatic dissemination of melanoma have been identified. Expression of these molecules has been studied in primary melanoma and correlated with prognosis. Moreover, several tumor suppressors and oncogenes have been shown to be involved in melanoma pathogenesis, including CDKN2A, PTEN, TP53, RAS, and myc, but have not been related to melanoma subtypes or validated as prognostic markers (Bosserhoff 2006). In the past, an increase in the number of positive tumor cells for Ki67, cyclin A, cyclin D, MMP-2, integrins beta1 and beta3 or osteonectin, as well as the decrease in p16, p27, and Melan A were considered as factors of poor prognosis in melanoma. However, only a small subset of these proteins has a prognostic value independent of tumor thickness. With the development of high-throughput technologies for analyzing global molecular profiles of cancer, previously unknown candidate genes involved in melanoma have been discovered, such as Wnt-5A and B-raf.

Table 5.9 Classification of biomarkers of melanoma

Genes
B-raf
Oncogenes: CDKN2A, myc, RAS
Tumor suppressors: PTEN, TP53
Wnt-5A
Protein biomarkers
Cell cycle-associated proteins: cyclin A, B, C, D
Matrix metalloproteinases (MMP)-1 and -9
Melan A
Melanoma-inhibiting activity (MIA)
Melastatin
p16
p27
S100B
Methylation biomarkers
MicroRNA biomarkers
Suppressed natural killer (NK) cell function
NKG2D
CD158a
CD158b
Serum biomarkers
Lactic dehydrogenase (LDH)
Melanoma cell adhesion molecules: soluble intracellular adhesion molecule 1 (sICAM-1)
Melanoma-inhibiting activity (MIA)
Melanocyte lineage/differentiation antigens: S100B
TA90-immune complex (TA90IC)
YKL-40
Imaging biomarkers
DCE-MRI

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YKL-40 is a growth factor for connective tissue cells and stimulates migration of endothelial cells. Cancer cells, macrophages, and neutrophils secrete YKL-40. Elevated serum YKL-40 is an independent prognostic factor for poor survival in patients with metastatic melanoma (Schmidt et al. 2006).

The first unbiased systematic effort to determine methylation biomarkers in melanoma by mapping chemical modifications of DNA in the melanoma genome has revealed several novel genes regulated by promoter methylation that were not described in cancer cells before (Koga et al. 2009). Discovery of new methylation biomarkers will help develop more effective treatment strategies to fight this disease.

The results of collaborative study by Genta Inc. and the Melanoma Group of the European Organization for the Research and Treatment of Cancer confirmed the strong relationship between patient survival and a biomarker that was prospectively studied in the randomized phase III trial of Genasense® (oblimersen sodium) injection in patients with advanced melanoma. The results of this trial showed that multiple outcomes, including overall survival, progression-free survival, overall response, and durable response, were strongly associated with blood levels of lactic dehydrogenase (LDH), a biomarker that was a prospectively defined stratification factor.

Currently, no biomarker to improve risk stratification is part of recommended clinical practice. Although numerous biomarkers candidates have been identified, their relevance to melanoma progression, clinical outcome, and the selection of optimal treatment strategies still needs to be established. A more accurate, therapeutically predictive classification of human melanomas and selection of patient populations that would profit from therapeutic interventions are among the major challenges expected to be addressed in the future (Gogas et al. 2009). Biomarker identification and validation will provide a rapidly changing molecular view of melanoma, a strategy that is necessary for developing truly stratified or personalized prevention or management (Ugurel et al. 2009).

5.5.14 Nasopharyngeal Carcinoma Biomarkers

Nasopharyngeal carcinoma (NPC) is a rare malignancy in most part of the world, and it is one of the most poorly understood and commonly misdiagnosed diseases. It is highly prevalent in southern Asia where the disease occurs at a prevalence about a 100-fold higher compared with other populations not at risk. As one of the most common cancers among Chinese or Asian ancestry, it poses one of the serious health problems in southern China where an annual incidence of more than 20 cases per 100,000 is reported (Cho 2007). Men are twice as likely to develop NPC as women. The rate of incidence generally increases from ages 20 to around 50. The etiology of NPC is thought to be associated with a complex interaction of genetic, viral (Epstein–Barr virus [EBV]), environmental, and dietary factors. Diagnosis of NPC at an early disease stage is important for successful treatment and improving the outcome of patients. Biomarkers of NPC and potential applications are shown in Table 5.10.

5.5.14.1 Proteomic Biomarkers of Nasopharyngeal Cancer

The use of serum protein profiles and a classification tree algorithm have been explored to distinguish NPC from noncancer control. Serum samples were applied to protein chips to generate mass spectra by SELDI-TOF MS and protein peak identification/clustering were performed using the Biomarker Wizard software

Table 5.10 Biomarkers of nasopharyngeal carcinoma and potential applications

Biomarkers discovered by use of proteomic technologies
Annexin A2
Basic transcription factor 3: signaling target
Ceruloplasmin: enhanced levels are normalized after positive response to therapy
Fibronectin: diagnosis
Heat shock protein 27: signaling target
Inter-α-trypsin inhibitor precursor: diagnosis
Mac-2-binding protein: diagnosis
Plasminogen activator inhibitor 1: diagnosis
Platelet factor-4: monitoring of response to treatment
Porin: signaling target
Serum amyloid: ProteinChip analysis to monitor relapse of NPC
Stathmin: signaling target
miRNA biomarkers of nasopharyngeal cancer
Tumor suppressor genes as molecular biomarkers of targeted therapies
THY1: associated with lymph node metastatic potential of NPC
BLU/ZMYND10: downregulated in NPC
GADD45G: its response to environmental stresses is disrupted epigenetically in NPC
14-3-3-σ gene product: upregulated by p53 in response to DNA damage and downregulated in NPC
Gene expression biomarkers as targets for therapy
Death-associated protein kinase (DAPK): loss of expression is associated with promoter region methylation in NPC. Potential reactivation by 5-*aza*-2′-deoxycytidine
EGFR: silencing by RNAi reduces the proliferation of NPC cells
Survivin: role in resisting apoptosis in NPC was confirmed by RNAi
Molecular biomarkers for prognosis and monitoring response to treatment
Endothelin-1: pretreatment plasma levels for predicting posttreatment failure in advanced NPC
Epstein–Barr virus (EBV): antibodies and EBV DNA are useful for the early detection, monitoring, and prognosis of NPC
Heparanase: overexpression is inversely correlated with survival of NPC patients
Tiam1: overexpression correlates with invasion and metastasis of NPC
IL-8 receptor A: overexpression in tumor cells indicates poor prognosis
VEGF: overexpression is associated with poor prognosis of NPC

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(Wing Yuen Ho et al. 2006). The results suggest that SELDI-TOF MS serum protein profiles could discriminate NPC from noncancer. The combination of serum protein profiles with an Epstein–Barr virus antibody serology test could further improve the accuracy of NPC screening.

Among the biomarkers under investigation, a promising one is Bmi-1. The Bmi-1 oncoprotein regulates proliferation and oncogenesis in human cells. Its overexpression leads to senescence bypass in human fibroblasts and immortalization of human mammary epithelial cells. One study has reported that compared with normal nasopharyngeal epithelial cells (NPEC), Bmi-1 is overexpressed in NPC cell lines (Song et al. 2006). Bmi-1 was also found to be overexpressed in 38.7 % NPC tumors by immunohistochemical analysis. In contrast to NPC, there was no detectable

expression of Bmi-1 in noncancerous nasopharyngeal epithelium. Moreover, high Bmi-1 expression positively correlated with poor prognosis of NPC patients. Overexpression of Bmi-1 leads to bypass of senescence and immortalization of NPECs, which normally express p16(INK4a) and exhibit finite replicative life span. Overexpression of Bmi-1 in NPECs led to the induction of hTERT activity and reduction of p16(INK4a) expression. These findings suggest that Bmi-1 plays an important role in the development and progression of BMC and that Bmi-1 is a valuable marker for assessing the prognosis of NPC patients.

5.5.14.2 miRNA Biomarkers of Nasopharyngeal Carcinoma

Use of highly sensitive microarray-based procedures has enabled the identification of eight miRNAs showing robust differential expression between laser capture-microdissected NPCs and normal healthy nasopharyngeal epithelial samples (SenGupta et al. 2008). In particular, miRNA mir-29c was expressed at one-fifth the levels in tumors as in normal epithelium. In NPC tumors, the lower mir-29c levels correlated with higher levels of multiple mRNAs whose 3′ UTRs can bind mir-29c at target sequences conserved across many vertebrates. In cultured cells, introduction of mir-29c downregulated these genes at the level of mRNA and inhibits expression of luciferase encoded by vectors having the 3′ UTRs of these genes. Most of the mir-29c-targeted genes identified encode extracellular matrix proteins, including multiple collagens and laminin γ1, which are associated with tumor cell invasiveness and metastatic potential, prominent characteristics of NPC. Thus, eight miRNAs are differentially expressed in NPC and are involved in regulating genes involved in metastasis.

NPC is associated with EBV infection, which was the first human virus found to encode miRNAs. EBV-encoded protein LMP1 is believed to be a key factor in NPC development. EBV miRNAs plays a role in regulating LMP1 downstream signaling to promote cancer development (Lo et al. 2007).

5.5.15 Oral Cancer Biomarkers

Oral cancer is a major global threat to public health with 300,000 new cases diagnosed worldwide on an annual basis. Oral cancer development is a tobacco-related multistep and multifocal process involving field carcinogenesis and intraepithelial clonal spread. The morbidity and mortality rates of this devastating disease have not improved in decades. Biomarkers of genomic instability, such as aneuploidy and allelic imbalance, can accurately measure the cancer risk of oral premalignant lesions or intraepithelial neoplasia (IEN). Retinoid-oral IEN studies (e.g., retinoid acid receptor-beta, p53, genetic instability, LOH, and cyclin D1) have advanced the overall understanding of the biology of intraepithelial carcinogenesis and preventive agent molecular mechanisms and targets, important advances for monitoring

preventive interventions, assessing cancer risk, and pharmacogenomics. However, there was a lack of plasma biomarker for detecting oral cancer.

A bead-based affinity-fractionated proteomic method has been developed to discover a novel plasma biomarker for oral cancer (Cheng et al. 2005). Affinity purification of heparinized plasma with magnetic beads and MALDI-TOF MS analysis were used to screen potential oral cancer markers. MS protein profiles were compiled for patients with oral cancer and compared with profiles from healthy controls. The spectra were analyzed statistically using flexAnalysis and ClinProt bioinformatic software. In each MS analysis, the peak intensities of interest were normalized with an internal standard (adrenocorticotropic hormone 18-39). For identification, affinity bead-purified plasma protein was subjected to MALDI-TOF/TOF analysis followed by Mascot identification of the peptide sequences and a search of the National Center for Biotechnology Information protein database. To optimize MALDI-TOF analysis based on the best discriminator of the cancer and control spectra, copper-chelated beads were used for plasma protein profiling. Six biomarkers that differentiated between cancer and control spectra were found. The 2,664-Da marker, identified as a fragment of the fibrinogen alpha-chain, had the highest sensitivity (100 %) and specificity (97 %) for cancer suggesting that it may be a clinical useful biomarker.

Both IL-8 and IL-1β are expressed at significantly higher levels in oral squamous cell carcinoma subjects than in the matched healthy control subjects. Luminex xMAP single-plex and multiplex assays are as effective as ELISA assays for quantification of biomarker proteins in saliva (Arellano-Garcia et al. 2008).

5.5.16 Ovarian Cancer Biomarkers

Ovarian cancer is the fourth leading cause of cancer deaths among women in the USA, despite its relatively low incidence of 50 per 100,000. On average, US women have a 2 % risk of developing ovarian cancer by age 70. Risk factors for ovarian cancer include previous breast cancer, family history of breast or ovarian cancer, and hereditary nonpolyposis CRC. Thus, approximately ten million women in the USA are at risk for the development of ovarian cancer and should be tested for it. Most women are diagnosed with ovarian cancer in late-stage disease and have a 5-year survival rate of less than 30 %, but these rates soar to over 90 % if the disease is discovered when cancer still is localized to the ovaries.

Clinical diagnosis of ovarian tumors is difficult in the absence of physical symptoms as the ovaries are located deep in the abdomen. Unfortunately, current methods of diagnosis, such as transvaginal ultrasound, laparoscopy, or PET scan, are impractical for general testing as they are complex procedures and would pose a tremendous burden on the healthcare system. The need for an accurate yet simple test has prompted investigators to explore novel, rapid ways of detecting ovarian cancer. To be practical for testing the high-risk population, a rapid detection assay for ovarian cancer should have the possibility to be performed on an easily obtained

Table 5.11 Biomarkers of ovarian cancer

Serum CA-125
Serum lysophosphatidic acids
Serum protein biomarkers
Biomarker protein pattern analysis
HE4 (human epididymis protein 4)
Mesothelin
Oviduct-specific glycoprotein: a tissue-specific, early-stage marker for ovarian malignancies
Serum albumin-associated peptides and proteins
Mutation of genes
BRCA1
P53
Gene expression studies
Tissue array analysis of gene expression: Ccne1, Ran, Cdc20, and Cks1
Gene and protein expression: CLU, ITGB3, CAPG, and PRAME
Epitomic approach for ovarian cancer biomarkers in serum
Multimarker panel of ovarian cancer biomarkers: OvPlex™ (HealthLinx)

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specimen, such as a blood sample. An assay that requires a tissue biopsy, for example, would be ruled out as a convenient test. The assay must be robust, such that normal handling and transport of the specimen to the testing laboratory does not alter the analyte or biomarker being measured. The need for special sample processing not normally performed at the patient service center, such as flash-freezing or extraction of a particular molecular component, could make it difficult to adapt the test for routine use. In the laboratory, the process for testing the sample should be high throughput and automated. Several tests are in development for ovarian cancer. Biomarkers for ovarian cancer are listed in Table 5.11, and tests relevant to biomarkers are discussed in the following sections.

5.5.16.1 Epitomics Approach for Ovarian Cancer Biomarkers in Serum

A noninvasive screening test can significantly facilitate early detection of epithelial ovarian cancer. One study used a combination of high-throughput selection and array-based serological detection of many antigens indicative of the presence of cancer, thereby using the immune system as a biosensor (Chatterjee et al. 2006). This high-throughput selection involved biopanning of an ovarian cancer phage display library using serum immunoglobulins from an ovarian cancer patient as bait. Protein macroarrays containing selected antigen clones revealed several that interacted with immunoglobulins in sera from ovarian cancer patients but not with sera

from healthy women or patients having other benign or malignant gynecologic diseases. Sequence analysis data of these clones revealed different antigens. Among the markers, some known antigens were identified, including RCAS1, signal recognition protein-19, AHNAK-related sequence, nuclear autoantogenic sperm protein, Nijmegen breakage syndrome 1 (Nibrin), ribosomal protein L4, Homo sapiens KIAA0419 gene product, eukaryotic initiation factor 5A, and casein kinase II, as well as many previously uncharacterized antigenic gene products. Using these antigens on protein microarrays, the investigators trained neural networks on two-color fluorescent detection of serum IgG binding and found an average sensitivity and specificity of 55 % and 98 %, respectively. In addition, the top six of the most specific clones resulted in an average sensitivity and specificity of 32 % and 94 %, respectively. This global approach to antigenic profiling, epitomics, has applications to cancer and autoimmune diseases for diagnostic and therapeutic studies. Further work with larger panels of antigens should provide a comprehensive set of markers with sufficient sensitivity and specificity suitable for clinical testing in high-risk populations.

xMAP technology (Luminex) is able to analyze multiple proteins in a single drop of blood or serum from women with ovarian cancer. Testing of 450 serum samples for 46 biomarkers that had previously been correlated with ovarian cancer was able to identify a multimarker panel, comprised of 20 proteins that correctly recognized more than 98 % of serum samples from women with ovarian cancer, offering higher diagnostic power than any other assay for ovarian cancer. The goal of the company is to develop this screening assay into a diagnostic test to improve the early detection of ovarian cancer and to monitor therapeutic response and recurrence in women with the disease.

5.5.16.2 Gene Expression Studies in Ovarian Cancer

IHC has been used to monitor a subset of differently expressed candidate genes (Ahr, Paep, Madh3, Ran, Met, Mek1, Ccne1, Ccd20, Cks1, and Cas) in tissue arrays composed of serous ovarian tumors of different grades (0–3) and stages (I–IV). All biomarkers assayed presented differential protein expression between serous tumors of low and high grade (Ouellet et al. 2006). Significant differences in Ccne1 and Ran expression were observed in a comparison of low malignant potential and grade 1 tumor samples. In addition, irrespective of the grade, Ccne1, Ran, Cdc20, and Cks1 showed significant differences of expression in association with the clinical stage of disease. While high level of Ccne1 have previously been associated with poor outcomes, this study found that high level of either Ran or Cdc20 appear to be more tightly associated with a poor prognosis. The application of these biomarkers in both the initial diagnosis and prognostic attributes of patients with epithelial ovarian tumors should prove to be useful in patient management.

One study on patients with serous ovarian adenocarcinomas found that the gene and protein ITGβ3 (integrin beta 3) were significantly more expressed in tumors from survivors compared to tumors from deceased patients, but no significant differences were detected for the other three genes or proteins CLU, CAPG, and PRAME (Partheen et al. 2009). Therefore, loss of ITGβ3 expression in tumors from deceased patients and high expression in tumors from survivors could be used as a biomarker for patients with advanced serous tumors.

5.5.16.3 HE4 Protein in Urine as a Biomarker for Ovarian Cancer

Human epididymis protein 4 (HE4) has been described as a new biomarker for the early diagnosis of ovarian cancer. HE4 protein is overexpressed in ovarian carcinomas and can be detected in serum by an ELISA with sensitivity similar to CA-125 and higher specificity for malignant disease. HE4 is better than CA-125 as biomarker for the diagnosis of ovarian cancer and could be an important early indicator of the recurrence of the disease (Anastasi et al. 2010). HE4 can also be detected in the urine at a specificity level of 94.4 %, including 86.6 % of tumors at stage I/II, 89.0 % at stage III/IV, and including 90.5 % of patients with serous ovarian carcinoma (Hellstrom et al. 2010). Assaying urine for HE4 or mesothelin may detect early ovarian carcinoma more often than assaying serum. Antibodies to mesothelin and HE4 are more frequent in women with ovarian carcinoma or with certain types of infertility than in controls (Hellstrom and Hellstrom 2011). Their data indicate that measuring HE4 in urine may aid diagnosis and the monitoring of response to therapy. The authors anticipate that, within the next 5 years, a greater emphasis will be given to the fact that the different subtypes of ovarian carcinoma represent different types of disease. Each different type of disease will require a different diagnostic approach and more efforts will focus on high-grade serous ovarian carcinoma for which the clinical need is the greatest.

5.5.16.4 HtrA1 as a Biomarker of Response to Chemotherapy in Ovarian Cancer

Expression of HtrA1, which is frequently downregulated in ovarian cancer, influences tumor response to chemotherapy by modulating chemotherapy-induced cytotoxicity. Two anticancer agents, cisplatin and paclitaxel, increase the expression of the HtrA1 in ovarian carcinoma cells and thus induce cell death. Conversely, reduced HtrA1 expression reduced the effectiveness of cisplatin and paclitaxel. Patients with ovarian or gastric tumors expressing higher levels of HtrA1 showed a better response to chemotherapy compared to those with lower levels of HtrA1 expression. A study of the pathway by which HtrA1 mediates the ability of chemotherapeutic agents to kill cancer cells suggests that loss of HtrA1 in ovarian and gastric cancer may contribute to the development of resistance to chemotherapy (Chien et al. 2006).

5.5.16.5 Mutation of Genes in Ovarian Cancer

Sporadic ovarian tumors are the end result of a complex pathway involving multiple oncogenes and tumor suppressor genes, including HER-2/neu, K-ras, p53, BRCA1, and additional tumor suppressor genes on chromosome 17. Recent studies indicate that germline mutations of the BRCA1 gene confer a lifetime risk of approximately 45 % for ovarian cancer in families with multiple cases of such cancer (this gene is also involved in breast cancer). In the general population mutations in the BRCA1 gene occur in approximately 5 % of women in whom ovarian cancer is diagnosed before the age of 70 years. Protein truncation test outperforms single strand conformation polymorphism analysis for BRCA1 mutation detection in ovarian cancer.

Mutations at the p53 tumor suppressor gene locus are frequently associated with human ovarian carcinoma. A multiplex PCR screening assay has been used to amplify the complete p53 coding region from genomic DNA in a single step. Deletions and insertions were subsequently found in the newly established ovarian carcinoma cell lines.

5.5.16.6 Serum Biomarkers of Ovarian Cancer Prognosis

Serum CA-125, the most studied biomarker for ovarian cancer, is only expressed by 50–60 % of patients with early-stage disease and has a very limited value for prediction of ovarian cancer. CA-125 can be elevated by conditions other than cancer. Considerable efforts have been deployed to identify novel serum markers, yet no single biomarker has emerged as a serious competitor for CA-125. The relationship between survival and early changes in the serum level of the CA-125 antigen in patients with advanced ovarian cancer is not well defined. Among women in the general US population, simultaneous screening with CA-125 and transvaginal ultrasound compared with usual care did not reduce ovarian cancer mortality (Buys et al. 2011). Diagnostic evaluation following a false-positive screening test result was associated with complications and unnecessary surgery. Pretreatment CA-125 values do not correlate with survival, but the concentration of this tumor biomarker after initiation of therapy is a powerful independent prognostic factor. Reduction in the serum CA-125 concentration over the initial two cycles of platinum-based chemotherapy was shown to be a powerful independent predictor of survival for patients with suboptimal stage III or IV ovarian cancer (Markman et al. 2006). Patients without significant declines in CA-125 after two cycles of platinum-based chemotherapy have a particularly poor prognosis.

VEGF is a therapeutic target in ovarian cancer due to important role of angiogenesis in tumor progression. The tissue inhibitor of metalloproteinase 1 (TIMP-1) is also involved in tissue invasion and angiogenesis. High TIMP-1 and VEGF serum levels during first-line therapy of ovarian cancer patients predict poor prognosis (Mahner et al. 2010).

5.5.16.7 Serum Albumin-Associated Peptides and Proteins

A method has been developed to isolate albumin in its native state by solid-phase affinity capture. Albumin-associated proteins and peptides were separated by gel electrophoresis and subjected to iterative MS sequencing by microcapillary reversed-phase tandem MS (Lowenthal et al. 2005). This method was used to analyze pooled sera from a study on high-risk persons without cancer and ovarian cancer to demonstrate the feasibility of this approach as a method for discovery of biomarkers. Selected albumin-bound protein fragments were confirmed in human sera by Western blotting and immunocompetition. The predicted sequences were largely fragments derived from proteins with diverse biological functions. More than one-third of these fragments were identified by multiple peptide sequences, and more than one half of the identified species were in vivo cleavage products of parent proteins. An estimated 700 serum peptides or proteins were predicted that had not been reported in previous serum databases, and several proteolytic fragments of larger molecules were found to be cancer related. BRCA2, a 390-kDa low-abundance nuclear protein linked to cancer susceptibility, was represented in sera as a series of specific fragments bound to albumin.

5.5.16.8 Multiplex Assays for Biomarkers of Ovarian Cancer

Since no single biomarker is adequate for detection of ovarian cancer, attempts have been made to use multiplex assays. Univariate and multivariate statistical analyses applied to protein-profiling data obtained from serum samples of patients with ovarian cancer using protein biochips has led to the discovery of biomarker protein panels, which can distinguish serum samples from healthy controls and patients with either benign or malignant ovarian neoplasia. Two tumor biomarkers, CA-125 and HE4 (approved by FDA), are used to track whether chemotherapy is working or ovarian cancer is recurring. A onetime CA-125 test cannot screen seemingly healthy women because levels rise with benign cysts, endometriosis, and even normal menstruation, but Fujirebio's triage test uses HE4 and CA-125 to assess who most likely has a benign cyst and whose has cancer.

OvaSure (LabCorp) measures concentrations of leptin, prolactin, OPN, insulin-like growth factor II, macrophage inhibitory factor, and CA-125 by using a multiplex, bead-based, immunoassay system. OvaSure is a screening test for women at high risk of ovarian cancer that was developed by Yale University under a law that allows a single laboratory to offer testing without FDA review. Used on blood samples stored from cancer patients and healthy women, the test correctly identified cancer with a sensitivity of 95.3 % and a specificity of 99.4 % (Visintin et al. 2008). However, this does not prove that OvaSure can detect when cancer is forming.

MS pattern analysis is a potentially rewarding approach in that it utilizes the power of combined multiple biomarkers so that, in principle, discrimination accuracy is higher. Having reliable, discriminatory patterns obviates the need to identify and purify the biomarkers of interest and develop molecular assays for them.

This process can be quite tedious, especially if the biomarkers are in low concentration. Furthermore, MS pattern assays take advantage of the high resolving power and small sample volume requirement of MS. MS pattern analysis requires laboratories to develop new ways to continually affirm platform and sample integrity in the absence of biology-based means. Essay variability could arise from potential heterogeneity of molecules within a spot, which is why SELDI-TOF employs multiple desorptions from different positions within a spot.

Various studies have shown that a 3-biomarker panel could classify stage I/II ovarian cancer samples healthy control samples with 97 % specificity and 74 % sensitivity, compared to 97 % specificity and 54 % sensitivity when CA-125 alone was used to classify. Even though it is considerably better than use of CA-125, it does not meet the requirements for a screening test. The 3-biomarker panel would have greater value if used in conjunction with another complementary test. OvPlex™ (HealthLinx) first-generation ovarian cancer 3-biomarker panel was launched in Australia with diagnostic efficiency of 92.9 %. In the second-generation product, two new novel biomarkers were added (HTX005 and HTX010). A phase II biomarker trial on the second-generation 5-biomarker panel OvPlex™ increased the diagnostic efficiency of the panel to 98 % for early-stage diagnosis as compared with CA-125 with diagnostic efficiency of less than 60 % for early-stage detection (http://www.ovplex.com.au/).

5.5.16.9 Concluding Remarks on Biomarker-Based Tests of Ovarian Cancer

The multiple ovarian cancer detection tests under development are based upon different complementary technologies and disparate biomarkers, so in principle their combined use will provide higher accuracy. Suboptimal sensitivity of a detection assay can be compensated somewhat through regular testing of women at high risk; a convenient assay makes such routine testing less burdensome and increases patient compliance. These arguments suggest that an assay with even 95 % sensitivity and specificity should help in the management of ovarian cancer.

A major advantage of isolating discrete biomarkers is that immunoassays or other biomolecule-specific assays can be developed for their detection. Immunoassays are performed routinely in the clinical lab on automated platforms with high throughput and, as such, are more economical and practical than SELDI-TOF in its present form. However, development of sufficiently sensitive immunoassays would be required.

Molecular analysis of ovarian cancer, a highly heterogeneous disease, reveals a large degree of variability among patients, and understanding this variability may be key for the development of tests able to detect various phenotypes of the disease. These differences are multifactorial, and therefore, investigating tumor/host interactions such as immune responses and angiogenesis may translate into the next generation of biomarkers for early detection of ovarian cancer (Seiden 2009).

Table 5.12 Classification of biomarkers of pancreatic cancer

Classical markers
Carcinoembryonic (CEA)
Carbohydrate antigen (CA 19-9)
Mucin family (MUC)
Oncogenes: Ki-67, p53, and bcl-2
Serum biomarkers
Angiogenesis and growth factors
Miscellaneous serum markers: M2 pyruvate kinase, HSP27
Tissue biomarkers
Caveolin-1 (Cav-1)
Histone modifications
Fibroblast activation protein (FAP)
Maspin (serpin B5)
Pancreatic and duodenal homeobox-1 (PDX-1)
Tissue transglutaminase 2 (TG2)
Omic biomarkers
Genomics/transcriptomics: K-ras, HIF1α, bFGF, VEGF, and PDGFA
Pharmacogenetic biomarkers of response to gemcitabine: specific SNPs with prognostic value
Proteomics: UHRF1, ATP7A, aldehyde oxidase 1 (AOX1), alpha1-antichymotrypsin, human R protein
Pancreatic CSCs
Signaling pathways biomarkers
MAPK and ERK pathway
TGF-β signaling pathway
notch, Hedgehog, and Wnt signaling pathways
MicroRNA biomarkers
miR-21, miR-210, miR-155, and miR-196a

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5.5.17 Pancreatic Cancer Biomarkers

The survival rate of pancreatic cancer patients is the lowest among those with common solid tumors, and early detection is one of the most feasible means of improving outcomes. Because of the complex pathophysiology of pancreatic cancer, sensitive and specific biomarkers are also required. Currently, there are no specific biomarkers for pancreatic cancer diagnosis, but extensive genomic/transcriptomic and proteomic studies are being carried out to find candidate biomarkers and contribute to high-throughput systems for large cohort screening. For improvement of early diagnosis, only a panel of soluble biomarkers could provide the appropriate combination between high sensitivity and specificity (Tanase et al. 2009). A classification of biomarkers is shown in Table 5.12, and some examples of proteomic and microRNA biomarkers will be described in the following text.

5.5.17.1 Discovery and Validation of Pancreatic Cancer Biomarkers

One project has created a compendium representing the first step in tackling biomarkers for pancreatic cancer in a global and systematic fashion (Harsha et al. 2009). It is already being used by a consortium of investigators who are developing antibodies against the 60 most promising targets. This compendium also included data on other, less common subtypes of pancreatic cancer. Because of the mRNA-based methods, especially DNA microarrays, 74 % of the molecules in this compendium are based solely on mRNA evidence. The data requires subsequent validation by other methods. Further, several high-throughput studies carried out to identify genes that are differentially expressed in pancreatic cancer have used tissues that are not microdissected to separate cancer from stroma. Thus, it is unclear in many instances if the observed difference in the expression of a particular gene originates in the stroma or in cancer cells. This further underscores the importance of validating these observations using alternative methods by targeted studies.

5.5.17.2 Cancer Stem Cells as Biomarkers of Pancreatic Cancer

There is increasing evidence that pancreatic cancer is hierarchically organized and sustained by a distinct subpopulation of CSCs. CSCs constitute a distinct subpopulation in the tumor and are considered to drive both tumorigenesis and metastasis; these cells are thought to be highly resistant to standard treatment modalities. Pancreatic CSCs are considered to be prognostic or predictive biomarkers as well as potential targets for therapy of pancreatic cancer (Sergeant et al. 2009). Direct evidence for the CSC hypothesis has emerged from mouse models suggesting that specific targeting of these cells is possible and therapeutically relevant (Hermann et al. 2009).

5.5.17.3 Histone Modifications Used as Biomarkers in Pancreatic Cancer

Measuring levels of specific histone modifications within cells has previously shown that low cellular levels of particular histones could determine which prostate cancer patients were more likely to suffer a recurrence and which patients with lung and kidney cancers would experience poorer survival rates. An assay to detect histone modifications can now be used to predict prognosis and response to treatment in subsets of patients with pancreatic cancer (Manuyakorn et al. 2010). The scientists used tissues from a cohort of patients enrolled in the radiation therapy oncology group (RTOG) 9704 trial, a multicenter, phase III study of pancreatic cancer comparing adjuvant gemcitabine with 5-FU, and a separate cohort of patients with stage 1 or 2 pancreatic cancer. IHC was performed for histone H3 lysine 4 dimethylation (H3K4me2), histone H3 lysine 9 dimethylation (H3K9me2), and histone H3 lysine 18 acetylation (H3K18ac). Positive tumor cell staining for each histone modification was used to classify patients into low- and high-staining groups, which were

related to clinicopathologic parameters and clinical outcome measures. Low cellular levels of H3K4me2, H3K9me2, or H3K18ac were each significant and independent predictors of poor survival. Combined low levels of H3K4me2 and/or H3K18ac were the most significant predictor of overall survival. In subgroup analyses, histone levels were predictive of survival specifically for those patients with node-negative cancer or for those patients receiving adjuvant 5-FU but not gemcitabine in RTOG 9704. The investigators concluded that cellular levels of histone modifications define previously unrecognized subsets of patients with pancreatic adenocarcinoma with distinct epigenetic phenotypes and clinical outcomes and represent prognostic and predictive biomarkers that could form basis of clinical decisions, including the use of 5-FU chemotherapy. Further research in cell lines and animal models will determine what, if any, role the histone modifications have in causing the development of aggressive forms of pancreatic cancer. Uncovering the mechanism of how the histone modifications are associated with cancer development and/or progression may facilitate design of strategies to interfere with that process and form the basis for a targeted therapy or chemoprevention.

5.5.17.4 miRNA Biomarkers of Pancreatic Cancer

A miRNA expression signature that is associated with pancreatic cancer has been identified (Lee et al. 2007). This has been accomplished with the application of real-time PCR profiling of over 200 miRNA precursors on specimens of human pancreatic adenocarcinoma, paired benign tissue, normal pancreas, chronic pancreatitis, and pancreatic cancer cell lines. Hierarchical clustering was able to distinguish tumor from normal pancreas, pancreatitis, and cell lines. An algorithm correctly classified all tumors. One hundred miRNA precursors were aberrantly expressed in pancreatic cancer or desmoplasia, including miRNAs previously reported as differentially expressed in other human cancers (miR-155, miR-21, miR-221, and miR-222) as well as those not previously reported in cancer (miR-376a and miR-301). Most of the top aberrantly expressed miRNAs displayed increased expression in the tumor. Reverse transcription in situ PCR showed that three of the top differentially expressed miRNAs (miR-221, miR-376a, and miR-301) were localized to tumor cells and not to stroma or normal acini or ducts. Aberrant miRNA expression may offer new clues to pancreatic tumorigenesis and may provide diagnostic biomarkers for pancreatic adenocarcinoma.

Scientists at Asuragen Inc. have used microarray and qRT-PCR platforms to identify miR-196a and miR-217 as the top biomarker candidates that distinguish pancreatic ductal adenocarcinoma (PDAC) from chronic pancreatitis. The qRT-PCR assay developed using this miRNA signature was validated using FFPE pancreatic blocks and achieved 95.24 % sensitivity and 94.87 % specificity. Early feasibility experiments showed that the assay can also be successfully used to identify PDAC in low tissue yielding clinical specimens, such as fine needle aspirate biopsies. In addition, interrogation of microdissected populations of normal, premalignant, and malignant cells revealed that miR-196a is specific to PDAC cells and

can be detected as early as in PanIn-2 precursor lesions. Ongoing efforts will assess whether elevated expression of miR-196a in pancreatic tissue may enable earlier identification of patients at high risk to develop PDAC in the future.

A modified protocol has been used to isolate and quantify plasma miRNAs from heparin-treated blood to show that miRNA in plasma can differentiate pancreatic adenocarcinoma patients from healthy controls (Wang et al. 2009). This was used to profile four miRNAs—miR-21, miR-210, miR-155, and miR-196a—all implicated in the development of pancreatic cancer with either proven or predicted target genes involved in critical cancer-associated cellular pathways. Of these, miR-155 has been identified as a candidate biomarker of early pancreatic neoplasia, whereas elevated expression of miR196a has been shown to parallel progression of disease. The results revealed a sensitivity of 64 % and a specificity of 89 % with the analyses of plasma levels for this panel of four miRNAs. The miRNA panel could detect more than 60 % of the pancreatic cancer cases. These observations, although a "proof of principle" finding at this time, show the feasibility of developing plasma miRNA profiling as a sensitive and specific blood-based biomarker assay for pancreatic cancer that has the potential of translation to the clinic with additional improvements in the future. This biomarker search is being expanded by taking a genome-wide approach to finding characteristic miRNAs in pancreatic cancer using miRNA microarrays.

5.5.17.5 Parathyroid Cancer Biomarkers

Parathyroid carcinoma (PC) is a rare malignancy and causes primary hyperparathyroidism (HPT) with high morbidity and mortality in advanced cases usually resulting from intractable hypercalcemia. Inactivation of the HRPT2/CDC73 gene, encoding the putative tumor suppressor protein parafibromin and discovered in the context of the hyperparathyroidism-jaw tumor (HPT-JT) syndrome, is a common, somatic event in most parathyroid cancers. Approximately 25 % of patients with apparently sporadic parathyroid cancer carry germline HRPT2/CDC73 mutation (Sharretts et al. 2010). Therefore, germline DNA analysis for HRPT2/CDC73 mutation is recommended in all patients with parathyroid cancer because of the potential benefit for first-degree relatives, who should nevertheless undergo serum calcium screening.

PC is usually not recognized preoperatively and is often not conclusively identified during surgery. There is a need for biomarkers of PC. Currently, several issues still need to be addressed, such as the lack of common criteria for the histopathological diagnosis of PC and preoperative diagnosis. The latter is important for the surgeon in deciding for a complete resection of all cancerous tissue at the time of the initial surgery with the increased likelihood of a cure (Falchetti et al. 2012).

5.5.17.6 Proteomic Biomarkers of Pancreatic Cancer

Expression Difference Mapping experiments with serum samples from patients with pancreatic adenocarcinoma compared to those with nonmalignant pancreatic

diseases and healthy controls have shown that the discriminative power of the resulting marker panels was superior to the established CA 19-9 marker and the best results were achieved by using combined panels of SELDI markers together with CA 19-9 (Koopmann et al. 2004).

In conventional practice, the use of CA 19-9 levels and imaging techniques is not optimal for detecting small pancreatic lesions. However, quantitative proteomics has shown great potential for the study of cancer and has opened new opportunities to investigate crucial events underlying pancreatic tumorigenesis and to exploit this knowledge for early detection and better intervention. Isotope-coded affinity tag technology for proteomic analysis of human cancer tissue has been used to identify differentially expressed proteins in pancreatic cancer (Chen et al. 2005).

Proteomic analysis of pancreatic juice has revealed hepatocarcinoma–intestine–pancreas/pancreatitis-associated protein (HIP/PAP) and protein that is 85 % identical to HIP/PAP, which has been designated as PAP-2 (Gronborg et al. 2004). The proteins identified in this study could be directly assessed for their potential as biomarkers for pancreatic cancer by quantitative proteomic methods or immunoassays.

One study has compared plasma proteomes between pancreatic cancer patients and sex- and age-matched healthy controls using surface-enhanced laser desorption/ionization coupled with hybrid quadrupole time-of-flight MS (Honda et al. 2005). A discriminating proteomic pattern was extracted from the data using a support vector machine learning algorithm and was applied to two validation cohorts. A set of four mass peaks most accurately discriminates cancer patients from healthy controls with sensitivity of 97.2 % and specificity of 94.4 %. When combined with CA 19-9, 100 % of pancreatic cancers, including early-stage (stages I and II) tumors, were detected. Although a multi-institutional large-scale study will be necessary to confirm clinical significance, the biomarker set identified in this study may be applicable to using plasma samples to diagnose pancreatic cancer.

A significant association was found between low expression of vascular endothelial growth factor-1 receptor (FLT-1) and both poor prognosis and advanced stage, suggesting that tumor expression of this VEGF receptor is a marker of less aggressive disease (Chung et al. 2006b). The development of antibody microarrays for molecular profiling provides insights into the nature of serum protein alterations in pancreatic cancer patients and shows the potential of combined measurements to improve sample classification accuracy.

A specific cell receptor, the RON receptor tyrosine kinase, is a member of the MET proto-oncogene family and is important for cell proliferation, differentiation, and cancer development. RON receptor is overexpressed in pancreatic cancer cells suggesting that the receptor may also contribute to the disease's development (Thomas et al. 2007). RON receptor was active in 93 % of pancreatic IEN, an early form of pancreatic duct cancer. In addition, the receptor was present in 79 % of primary pancreatic cancers and 83 % of metastatic cancers. RON receptor's signaling pathways could be a key factor contributing to pancreatic cancer progression. If the receptor could be blocked, it would provide a new target for RON-directed therapies that are more effective in treating this cancer.

One study has searched for pancreatic cancer biomarkers by use of high-resolution MS and acrylamide isotopic labeling in the plasma proteome of mice genetically engineered to develop cancers that closely resemble human pancreatic tumors (Faca et al. 2008). Finally, using blood samples collected during a clinical trial, the CARET (a cancer prevention study), the researchers in this study showed that the measurement of five of the proteins present in increased amounts at an early stage of tumor development in the mouse model discriminated between people with pancreatic cancer and matched controls up to 13 months before cancer diagnosis. Such proteins could be used in screening blood tests for early and more accurate detection of cancer.

5.5.18 Prostate Cancer

Prostate cancer is the most common cause of death from cancer in men over the age of 75. Over 230,000 new prostate cancer cases and 30,000 prostate cancer deaths are estimated in the USA per year. This makes prostate cancer the most commonly diagnosed cancer and the second leading cause of cancer-related deaths in men in the USA. Various biomarkers of cancer of the prostate are listed in Table 5.13.

5.5.18.1 Adipose Tissue-Derived Biomarkers of Obesity-Related Prostate Cancer

Obesity (adiposity) is associated with prostate cancer, particularly with its accelerated progression. Adipose factors including adiponectin, leptin, IGF, and IL-6 are molecular mediators between prostate cancer and obesity. Researchers at the Medical College of Wisconsin have shown that leptin and other adipose factors such as IGF-1, IGF-II, TNF-α, and IL-6 stimulate prostate cancer cell growth and subsequent blockage of these adipose factors will suppress androgen-independent prostate cancer cell growth and increase survival. These adipose factor functions can be blocked by inhibiting their expression, the expression of their cell surface receptors, or by inhibiting the binding of these cytokines to their receptors. Adipokines may contribute to the molecular basis for the association between obesity and prostate cancer, but the complex pathophysiology of both these disease states requires further studies (Mistry et al. 2007).

5.5.18.2 B7-H3 as Biomarker of Prostate Cancer

Until now there were no strongly predictive molecules for prostate cancer. PSA and PSMA are useful for diagnosis of prostate cancer. However, PSA tends to leave prostate cancer cells and migrate throughout the body, making it a poor target for therapy. Mayo Clinic researchers have identified B7-H3 as the first immune molecule that appears to play a role in prostate cancer development and in predicting

Table 5.13 Biomarkers of prostate cancer

Antigens
Prostate-specific antigen
Prostate-specific membrane antigen
Adipose tissue-derived biomarkers of obesity-related prostate cancer
Adipokines
Metabolomic biomarkers of prostate cancer
Sarcosine
Prostate biomarkers in body fluids
Biomarker in semen
Biomarker in urine: annexin 3; EPCA-2, sarcosine
Exosomes as biomarkers in serum and urine
mRNA biomarkers in serum and urine
HAAH in serum
Huntingtin interacting protein 1: autoantibodies in serum
Prostasomes in serum
Serum protein fingerprinting
Biomarkers of prostate cancer in biopsy specimens
Expression of proteins: hepsin and PIM1
Id proteins expression in prostate cancer
Genes relevant to diagnosis of prostate cancer
Kallikrein gene polymorphisms: KLK2, KLK3, etc.
Loss of p27 as predictor of recurrence of prostate cancer
Detection of DD3 (PCA3) gene in urine
Genetic biomarkers of prostate cancer
Tests for prostate cancer based on genetic dislocations
Tests for prostate cancer based on gene expression
Epigenetic biomarkers of prostate cancer
miRNA biomarkers of prostate cancer
Integrative genomic and proteomic profiling of prostate cancer

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cancer recurrence and progression after surgery (Roth et al. 2007). Expression levels of B7-H3 in prostate cancer specimens of men treated for clinically localized prostate cancer were correlated with pathological indicators of aggressive cancer as well as clinical outcome, and B7-H3 was uniformly and aberrantly expressed by adenocarcinomas of the prostate. B7-H3 is expressed by benign prostatic epithelia,

although at a more reduced level relative to neoplastic tissue. Because B7-H3 kills or paralyzes immune cells that are trying to attack the cancer, it encompasses a novel diagnostic, prognostic, and even a therapeutic target for the clinical management of prostate cancer. B7-H3 represents an independent predictor of prostate cancer progression following surgery. This discovery will allow physicians to individualize treatment and observation plans for prostate cancer patients. Being able to tell a patient his specific risk after surgery, and perhaps even prior to surgery, will be a huge step forward. Evaluation of B7-H3 levels in prostate biopsies from patients may help to determine which patients may benefit from a watchful waiting strategy versus early aggressive treatment. Using this molecular signature will facilitate, for the first time, a truly individualized approach to prescribing the most appropriate therapy for a given patient, i.e., personalized medicine.

5.5.18.3 Cancer Genetics-Guided Biomarker Signatures of Prostate Cancer

A two-stage strategy has been described for the discovery of serum biomarker signatures corresponding to specific cancer-causing mutations and its application to prostate cancer in the context of the commonly occurring PTEN tumor suppressor gene inactivation (Cima et al. 2011). In the first stage of this approach, 775 N-linked glycoproteins were identified from sera and prostate tissue of wild-type and PTEN-null mice. Using label-free quantitative proteomics, it was shown that PTEN inactivation leads to measurable perturbations in the murine prostate and serum glycoproteome. In the second stage, following bioinformatic prioritization, targeted proteomics was applied to detect and quantify 39 human ortholog candidate biomarkers in the sera of prostate cancer patients and control individuals. The resulting proteomic profiles were analyzed by machine learning to build predictive regression models for tissue PTEN status and diagnosis and grading of prostate cancer. This approach suggests a general path to rational cancer biomarker discovery and initial validation guided by cancer genetics and based on the integration of experimental mouse models, proteomic-based technologies, and computational modeling.

5.5.18.4 Detection of Prostate Cancer Biomarkers in Urine

Urine is considered to be an attractive body fluid in which to pursue the identification of novel biomarkers of prostate cancer. Some proteomic techniques including MS and the newer, quantitative proteomic strategies have been applied to novel urinary biomarker identification in prostate malignancy.

A urine test for prostate cancer based on detection of differential display code 3 or DD3(PCA3) gene. PROGENSA™ PCA3 (Gen-Probe) is the first gene-based, adjunctive screen for prostate cancer. PCA3 is overexpressed only in malignant prostate tissue and not in any other normal human tissue, including breast, bladder, testis, gastrointestinal organ, and musculoskeletal tissue. A quantitative RT-PCR

assay for PCA3 has been tested and showed a 66-fold upregulation of PCA3 in patients with prostate cancer. This assay would be a potentially valuable tool for the detection of malignant cells in blood, urine, or other clinical specimens, and it could have important implications for the earlier diagnosis and molecular staging of prostate cancer (Marks et al. 2007). The test offers advantages over PSA testing, the current standard for initial prostate cancer screening in conjunction with a digital rectal exam. It has great potential in reducing the number of unnecessary prostate biopsies.

Bostwick Laboratories is also developing a urine-based test for prostate cancer risk, PCA3Plus, which detects PCA3 in cells that are shed in urine. PCA3Plus tests have been validated but not approved by the FDA. The test is based on reagents manufactured by Gen-Probe. Gen-Probe is funding a study at the Center for Prostate Disease Research (CPDR) to create a new generation of highly specific gene panels in urine for diagnosis of prostate cancer. CPDR is currently trying to assess the clinical utility of expression alterations of a novel prostate-specific gene, PCGEM1, and the ETS-related gene, ERG, in prostate patients. CPDR's oncogenes may be studied in combination with other prostate cancer biomarkers owned by Gen-Probe, including PCA3 and AMACR.

A multiplex panel of urine transcripts was shown to outperform PCA3 transcript alone for the detection of prostate cancer (Laxman et al. 2008). The test is based on the finding that gene fusions—pieces of chromosomes that trade places with each other, causing two genes to stick together—are common in prostate cancer, and that by overriding molecular switches that turn off excess growth, they may be the causative factor in some forms of the disease. The investigators considered six gene fusions in addition to PCA3 as putative prostate cancer biomarkers: TMPRSS2, which fuses with either ERG or ETV1, two genes known to be involved in several types of cancer and another five genes that fuse on to ERG or ETV1 to cause prostate cancer. The expression of these seven biomarkers was measured in sedimented urine using quantitative PCR from patients presenting for biopsy or radical prostatectomy. Univariate analysis showed that increased GOLPH2, SPINK1, and PCA3 transcript expression and TMPRSS2–ERG fusion status were significant predictors of prostate cancer. Multivariate regression analysis showed that a multiplexed model, including these biomarkers, outperformed serum PSA or PCA3 alone in detecting prostate cancer. The urine test that screens for the presence of four different RNA molecules accurately identified over 75 % of patients (5 % better than with PCA3 alone) who were later found to have prostate cancer and was 61 % effective in ruling out disease in other study participants. These results provide the basis for the development of highly optimized, multiplex urine biomarker tests for more accurate detection of prostate cancer.

Studies have shown that Annexin 3 (ANXA3) quantification in urine is a novel, noninvasive test with high specificity for prostate cancer. bioMérieux and ProteoSys are collaborating to develop a confirmatory urine test for prostate cancer based on Annexin 3. This will reduce the number of unnecessary biopsies. bioMérieux is also considering the development of treatment decision and prognostic applications for ANXA 3 (Köllermann et al. 2008).

5.5.18.5 Detection of Prostatic Intraepithelial Neoplasia

High-grade prostatic intraepithelial neoplasia (PIN) has been established as a premalignant lesion of the prostate that has a high potential to progress to invasive prostate cancer. Over 1.4 million prostate biopsies are performed in the USA every year to detect new cases of prostate cancer. High-grade PIN is found in an average of 9 % of prostate biopsies which represents an estimated 115,000 new cases of high-grade PIN diagnosed each year. Patients who are found to have high-grade PIN are at high risk of prostate cancer with up to 37 % of patients being later diagnosed with prostate cancer within 1 year.

PIN may prove to be an important diagnostic indicator of cancer of prostate. The development of assays for the accurate detection of PIN could be a component in the treatment of this disease. A proprietary panel of proPSA serum markers from Beckman Coulter may help diagnose the presence of PIN and the earliest progression to prostate cancer.

5.5.18.6 Epigenetic Biomarkers of Prostate Cancer

In contrast to genetic changes that may vary, certain epigenetic changes are highly consistent, in particular hypermethylation of a specific set of genes, and others regularly associated with progression, such as global DNA hypomethylation, certain chromatin modifications, and altered levels and composition of Polycomb complexes. Although changes in Polycombs and DNA methylation both accompany the progression of prostate cancer, they do not cause one another. However, they may contribute to establishing and maintaining an aberrant differentiation potential of prostate cancer-initiating cells. Global DNA hypomethylation in prostate cancer may relate to adaptive changes in several signaling pathways typical of this cancer type, including innate immunity responses. Adaptive changes in the expression and function of chromatin regulators required to diminish the dependency of prostate cancer cells on androgens may shape the epigenome, beyond individual genes regulated by the androgen receptor (AR) (Schulz and Hoffmann 2009). Because of their crucial role, epigenetic biomarkers may become highly useful for diagnostics and therapy of prostate cancer.

Hypermethylated DNA can be detected in body fluids from prostate cancer patients and may be a useful biomarker, although clinical performance varies between studies. Real-time PCR was used to measure four DNA methylation biomarkers: GSTP1 and three previously unreported candidates associated with the genes RASSF2, HIST1H4K, and TFAP2E in sodium bisulfite-modified DNA (Payne et al. 2009). Analysis of all biomarkers in urine DNA significantly discriminated prostate cancer from biopsy negative patients. The biomarkers provided information independent of PSA and may warrant inclusion in nomograms for predicting prostate biopsy outcome. The biomarkers' prostate cancer sensitivity was greater for urine than plasma DNA. The biomarker performances in urine DNA should next be validated in formal training and test studies.

5.5.18.7 Exosomes as Biomarkers of Prostate Cancer

Discovery of novel biomarkers for prostate cancer (PCa) in complex fluids, such as serum and urine, remains a challenge. Meanwhile, recent studies have reported that many cancer-derived proteins and RNAs are secreted through small vesicles known as exosomes, which are potential biomarkers for PCa (Duijvesz et al. 2011). Purification of prostate- and PCa-derived exosomes will enable us to profile exosomes, providing a promising source of protein and RNA biomarkers for PCa. This profiling will contribute to the discovery of novel biomarkers for the early diagnosis and reliable prognosis of PCa. Although the initial results are promising, further investigations are required to assess the clinical value of these exosomes in PCa.

5.5.18.8 Gene Expression Analysis of Prostate Cancer

Gene expression levels have been compared with two reference samples—normal prostate tissue from men with prostate cancer and prostate samples from men with no history of prostate disease. Expression of two proteins, hepsin (the membrane-spanning serine protease) and PIM1 (an oncogenic kinase), is significantly correlated with poor prognosis in prostate cancer. These biomarkers are unlikely to replace PSA in the clinic for routine screening, as taking a blood sample is far less invasive than a biopsy; however, when biopsy material is available, these cell-associated makers might be useful for identifying candidates for prostatectomy.

One novel prostate cancer gene identified by gene expression analysis is hepsin—a serine transmembrane protease that is overexpressed in cancer cells from both primary and metastatic tumors. Another gene, AMACR, is upregulated only in localized prostate cancer tumors, but not metastatic. This gene is more reliable than PSA for identifying aggressive prostate tumors; when gene expression patterns of additional prostate cancer patients were evaluated, AMACR expression alone can predict, which patients had aggressive prostate tumors, with 97 % specificity and 100 % sensitivity.

Quantitative RT-PCR assays show that a four-gene expression signature for prostate cancer cells (consisting of UAP1, PDLIM5, IMPDH2, and HSPD1) can detect Gleason grade 3 and grade 4 cancer cells in prostate tissue (Guyon et al. 2009). This may be useful as an adjunct test to the pathology examination of prostate tissue taken at biopsy or prostatectomy.

Significant and widespread differences in gene expression patterns exist between benign and malignant growth of the prostate gland. Gene expression analysis of prostate tissues should help to disclose the molecular mechanisms underlying prostate malignant growth and identify molecular biomarkers for diagnostic, prognostic, and therapeutic use.

5.5.18.9 Genetic Biomarkers of Prostate Cancer

Scientists have identified a common genetic biomarker that signals a 60 % increased risk of prostate cancer in men who carry it. Allele-8 of the microsatellite DG8S737, a variant on chromosome 8q24, is associated with prostate cancer in three case–control series of European ancestry from Iceland, Sweden, and the USA (Amundadottir et al. 2006). About 19 % of affected men and 13 % of the general population carry at least one copy, yielding a population attributable risk (PAR) of ~8 %. The association was also replicated in an African-American case–control group in which 41 % of affected individuals and 30 % of the population are carriers. This leads to a greater estimated PAR (16 %) that may contribute to higher incidence of prostate cancer in African-American men than in men of European ancestry. deCode plans to use the discovery to develop a genetic test that might help physicians decide how closely to follow men at high risk and how to treat prostate cancer cases. The study indicated that the variant might be associated with more aggressive forms of the disease. It is not clear whether the heightened risk comes from the variant itself or from another that lies nearby on chromosome 8.

5.5.18.10 Huntingtin Interacting Protein 1 Overexpression in Prostate Cancer

Huntingtin interacting protein 1 (HIP1) is an oncogene for prostate, colon, and brain cancers. In addition to human cancers, HIP1 is also overexpressed in prostate tumors from the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model. Autoantibodies to HIP1 developed in the sera of TRAMP mice with prostate cancer as well as in the sera from human prostate cancer patients have led to the development of an anti-HIP1 serum test in humans that had a similar sensitivity and specificity to the anti-alpha-methylacyl-CoA racemase (AMACR) and PSA tests for prostate cancer and when combined with the anti-AMACR test yielded a specificity of 97 % (Bradley et al. 2005). These data suggest that HIP1 plays a functional role in carcinogenesis and that a positive HIP1 autoantibody test may be an important serum marker of prostate cancer.

HIP1 provides a potential target for screening in tissue samples, and involvement of HIP1 in the clathrin trafficking pathway can also form the basis for a therapeutic target. HIP1 antibodies and several cell lines have been created that overexpress HIP1 at varying levels, which can be used for a high-throughput screen for therapeutic compounds that target HIP1. Potential applications include therapeutics based on antibodies, domain targeting by small molecules, and RNAi approaches.

5.5.18.11 Id Proteins Expression in Prostate Cancer

A major cause of treatment failure for prostate cancer is the development of androgen-independent metastatic disease. Id protein family, a group of basic helix-loop-helix

transcription factors, has been shown to be involved in carcinogenesis and a prognostic marker in several types of human cancers. A study has examined the expressions of four Id proteins, Id-1, Id-2, Id-3 and Id-4, in clinical prostate cancer specimens as well as nodular hyperplasia specimens by IHC (Yuen et al. 2006). The results indicate that these Id proteins may play a positive role in the development of prostate cancer. Differential Id proteins expressions may be a useful marker for poor prognosis, and Id-4 may be a potential prognostic marker for distant metastasis.

5.5.18.12 Identification of Prostate Cancer mRNA Biomarkers

RT-PCR differential display method has been used initially to identify mRNA transcripts differentially expressed in tumor versus patient-matched nontumor prostate tissue. Averaged differential expression (ADE) of pooled tissue samples was used to identify mRNA transcripts that are differentially expressed in most tumor specimens (Bai et al. 2007). Differentially expressed mRNA transcripts identified by ADE were fewer in number, but were expressed in a greater percentage of tumors (>75 %) than those identified by differential display of mRNA from individual patient samples. Differential expression of these mRNA transcripts was also detected by RT-PCR in mRNA isolated from urine and blood samples of prostate cancer patients. These findings demonstrate the principle that specific cDNA probes of frequently differentially expressed mRNA transcripts identified by ADE can be used for the detection of prostate cancer in urine and blood samples.

5.5.18.13 Integrative Genomic and Proteomic Profiling of Prostate Cancer

2D GE remains the most powerful method for analysis of cellular protein phenotype and may reveal gene regulations that cannot be detected on a genetic level (Hellstrom et al. 2007). Molecular profiling of cancer at the transcript level has become routine. Large-scale analysis of proteomic alterations during cancer progression has been a more daunting task. High-throughput immunoblotting has been used to interrogate tissue extracts derived from prostate cancer (Varambally et al. 2005). This approach identified 64 proteins that were altered in prostate cancer relative to benign prostate and 156 additional proteins that were altered in metastatic disease. An integrative analysis of this compendium of proteomic alterations and transcriptomic data was performed, revealing only 48–64 % concordance between protein and transcript levels. Importantly, differential proteomic alterations between metastatic and clinically localized prostate cancer that mapped concordantly to gene transcripts served as predictors of clinical outcome in prostate cancer as well as other solid tumors.

5.5.18.14 Kallikreins as Biomarkers of Prostate Cancer

Polymorphisms associated with prostate cancer include those in three genes encoding major secretory products of the prostate: KLK2 (encoding kallikrein-related peptidase 2; hK2), KLK3 (encoding PSA), and MSMB (encoding beta-microseminoprotein). PSA (hK3) is one of the human kallikreins and is the most used tumor biomarker for prostate cancer screening, diagnosis, prognosis, and monitoring. hK2, another prostate-specific kallikrein, has also been proposed as a complementary prostate cancer biomarker.

Genotyping of SNPs in "Cancer Prostate in Sweden 1" with independent replication in "Cancer Prostate in Sweden 2" study showed that a T allele at rs198977 in KLK2 was associated with increased cancer risk and a striking decrease of hK2 levels in blood (Klein et al. 2010). Based on this strong association, the investigators developed a model for predicting prostate cancer risk from standard biomarkers, rs198977 genotype, and rs198977 × hK2 interaction; this model had greater accuracy than did biomarkers alone.

Multiple kallikrein forms measured in blood can predict the result of biopsy in previously unscreened men with elevated PSA. A multivariable model can determine which men should be advised to undergo biopsy and which might be advised to continue screening, but defer biopsy until there was stronger evidence of malignancy (Vickers et al. 2008). OPKO Health Inc.'s novel panel of kallikrein biomarkers and associated algorithm for the detection of prostate cancer has been shown to predict the probability of cancer-positive biopsy in over 10,000 men suspected for prostate cancer. Use of this panel could eliminate unnecessary prostate biopsies, demonstrating a reduction of >50 %.

5.5.18.15 LCM for Diagnosis of Prostate Cancer

The proportion of unbound serum PSA (percent-free PSA) is reported to be lower in men with prostate cancer compared to men with benign prostates. The majority of immunoreactive PSA in serum is complexed to alpha-1-antichymotrypsin (ACT). LCM has been used to assess the bound versus free form of intracellular PSA in both benign and malignant epithelia procured from prostate tissue. Western blotting analysis of one-dimensional PAGE gels revealed that in the vast majority of intracellular tumor, normal PSA exists within cells in the "free" form. Binding studies showed that PSA recovered from LCM-procured cells retained the full ability to bind ACT and 2D PAGE Western analysis demonstrated that the PSA/ACT complex was stable under strong reducing conditions. Intracellular PSA, therefore, exists in the "free" form and that binding to ACT occurs exclusively outside of the cell.

PSA or histological examination of bulk tissue may not accurately reflect molecular events that take place in the actual ductal epithelium that change as a consequence of the malignant process in the prostate gland. Alternative proteomic-based approaches including LCM enable the identification of protein markers in the actual premalignant and frankly malignant epithelium.

The phenotype of a given cell type is ultimately determined by the composition and activation status of its proteins. Therefore, quantitative and qualitative proteomic measurement of normal and neoplastic prostate cells is an important experimental approach that will complement genomic DNA and gene expression analyses. The National Cancer Institute Prostate Group has been studying protein profiles of prostate cancer using tissue microdissection and two protein analysis methods: 2D PAGE and SELDI.

SELDI ProteinChip MS technology has been used for the rapid, reproducible, and simultaneous identification of four well-characterized prostate cancer-associated biomarkers: PSA (free and complexed forms), prostate-specific peptide, prostate acid phosphatase, and prostate-specific membrane antigen in cell lysates, serum, and seminal plasma. Proteins corresponding to the mass of these biomarkers could readily be captured and detected using either chemically defined or antibody-coated ProteinChip arrays. Several other proteins were found upregulated in cell lysates of pure populations of prostate cancer-associated cells procured by LCM when compared with mass spectra of normal cell lysates. Coupling LCM with SELDI provides tremendous opportunities to discover and identify the signature proteins associated with each stage of tumor development.

5.5.18.16 Loss of p27 as Predictor of Recurrence of Prostate Cancer

Also promising is the finding that expression of p27 in radical prostatectomy specimens correlates with biochemical recurrence. Loss of p27 (defined as absent expression in more than 70 % of the specimen) is an independent predictor of recurrence among all patients and among the subset with organ-confined and extracapsular disease. p27 can also predict outcome among patients with positive surgical resection margins. As with other biomarkers, major questions still to be addressed are the requirement for universal application with uniform scoring and the need for prospective studies in randomized clinical trials.

5.5.18.17 Microarray for Diagnosis of Prostate Cancer

Microarray technology has been used to analyze the molecular differences between tissue from unaffected individuals and those with benign prostatic hyperplasia (BPH), primary localized prostate cancer or metastatic hormone-refractory prostate cancer. Gene expression levels were compared with two reference samples—normal prostate tissue from men with prostate cancer and prostate samples from men with no history of prostate disease. Expression of two proteins, hepsin (the membrane-spanning serine protease) and PIM1 (an oncogenic kinase), is significantly correlated with poor prognosis in prostate cancer. These markers are unlikely to replace PSA in the clinic for routine screening, as taking a blood sample is far less invasive than a biopsy; however, when biopsy material is available, these cell-associated makers might be useful for identifying candidates for prostatectomy.

5.5.18.18 miRNA Biomarkers of Prostate Cancer

Optimized high-throughput miRNA expression profiling offers novel biomarker identification from prostate cancer biopsies enabling distinction of advanced and metastatic prostate cancers from pooled normal prostatic samples and from nonmalignant precursor lesions. Hierarchical clustering of the prostate tumor samples according to their miRNA expression can separate the carcinomas from the benign prostate hypertrophy and also further classified the carcinomas according to their androgen dependence (Porkka et al. 2007). This indicates that miRNAs expression profiling is a potential diagnostic and prognostic tool for prostate cancer.

miRNAs originating from human prostate cancer xenografts enter the circulation, are readily measured in plasma, and can robustly distinguish xenografted mice from controls. This concept extends to cancer in humans, where serum levels of miR-141 (a miRNA expressed in prostate cancer) can distinguish patients with prostate cancer from healthy controls (Mitchell et al. 2008).

A multiplex RT-qPCR method involving the purification of PCR products followed by uniplex analysis on a microfluidic chip was developed to evaluate and to identify serum miRNAs that help diagnosis and correlate with the prognosis of prostate cancer (Moltzahn et al. 2011). By profiling sera from healthy men and untreated prostate cancer patients with differing CAPRA scores, the investigators identified miRNA signatures including oncogenic and tumor-suppressive miRNAs that enabled diagnosis of prostate cancer and correlation with prognosis as well as cancer progression. Some dysregulated miRNAs are predicted to target kallikreins, and the miRNA-kallikrein axis of interaction provides a new factor in the pathogenesis of prostate cancer (White et al. 2012).

Extracellular miRNAs exist in different forms—associated with Ago2 proteins, loaded into extracellular vesicles (exosomes, microvesicles, or apoptotic bodies) or into high-density lipoprotein particles. Potential use of miRNAs from extracellular vesicles as biomarkers for prostate cancer is under consideration (Hessvik et al. 2013).

5.5.18.19 Prostate Cancer Biomarkers in Semen

The proportion of free and complex PSA in serum is used for differentiating between benign and malignant prostate disease. To further understand the physiological relationship between PSA in seminal plasma and blood, free prostate-specific antigen (fPSA) and complex prostate-specific antigen (cPSA) have been analyzed in blood and in seminal plasma in young healthy men (Savblom et al. 2005). fPSA in blood, but not cPSA, is associated to PSA in semen (approximately 17 % covariation). In blood cPSA, but not fPSA, increases with age in healthy men, which may reflect an increasing incidence of prostate disease.

A semen-based prostate cancer test, designed to improve on the accuracy of the PSA test, has identified a prostate cancer biomarker associated with human carcinoma antigen (HCA). When present along with HCA, the biomarker will form the basis of a new prostate cancer diagnostic test being developed by Tyrian Diagnostics.

Ambrilia Biopharma's PSP94 (Prostate Secretory Protein of 94 amino acids) immunoassay is a simple, potential diagnostic tool to determine the stage, grade, and aggressiveness of prostate cancer. PSP along with PSA and prostate acid phosphatase (PAP) are the three most abundant proteins in seminal fluid. Levels of PSA above 4 ng/mL of blood can signal prostate cancer, but not always. As prostate cancer advances, the serum PSP94 level is seen to initially increase but then drops in highly advanced cancer. Ambrilia's immunoassay measures the amount of free PSP94, bound PSP94, and PSP94-binding protein present in the serum. Studies have also shown that levels of PSP94 are hormone-independent and not affected by androgen or antiandrogen therapy. PSP94 assessments are therefore likely to be of use during periods of androgen ablation therapy.

5.5.18.20 PSA as Biomarker of Prostate Cancer

At present, measurement of serum PSA is the most useful biomarker for early detection, clinical staging, and monitoring. However, although on average men with prostate cancer have higher levels of PSA than healthy men or those with benign prostate diseases, there is a wide variation in levels throughout the population, which leads to false-positives and unnecessary biopsies. Also PSA test cannot reliably distinguish between prostate conditions such as BPH and cancer. A low PSA is not a guarantee of disease-free status, and an elevated PSA is frequently associated with a negative biopsy. Of the 25 million men tested for PSA, one million will undergo prostate biopsy based upon elevated levels of PSA. It is estimated that 75 % of these biopsies are unnecessary, a significant portion of which could be avoided if a more accurate diagnostic tool is available. A case–control study has shown that men at age 60 with a PSA level below 1 ng/mL, which is about half of all men, had a 0.2 % chance of death from prostate cancer, are at low risk of prostate cancer death, and may not need to be screened in the future (Vickers et al. 2010). The study also indicated that some men found to be at low risk may actually have prostate cancer; however, it is not likely to cause symptoms or shorten their life by the age of 85.

5.5.18.21 ProPSA as Biomarker of Prostate Cancer

ProPSA ($^{-2}$proPSA) is present in prostate tumor tissue but not in normal prostate, and higher levels in serum indicate development of an aggressive form of prostate cancer with an unfavorable prognosis. In a prospective cohort of men enrolled into expectant management for prostate cancer, serum and tissue levels of proPSA at diagnosis are associated with need for subsequent treatment (Makarov et al. 2009). The increase in serum proPSA/% free PSA (fPSA) might be driven by increased proPSA production from premalignant cells in the prostate benign adjacent areas. A prospective study has demonstrated that $^{-2}$proPSA provides improved discrimination between prostate cancer and benign disease in screened men with a PSA of 2.5–10 ng/mL and a negative digital rectal examination (Le et al. 2010).

5.5.18.22 Prostate Health Index

Prostate Health Index (PHI) involves combination of results of serum total PSA, fPSA, and $^{-2}$proPSA measured by the Beckman Coulter immunoassay. All the three should be analyzed simultaneously in the same serum sample. Values of PHI in general population are 33 and, if >44, are associated with 44 % risk of prostate cancer. As a simple blood test, it significantly improves on PSA in the selection of men for biopsy and is a major advance in prostate cancer risk assessment. Implementation of PHI in Prostate Cancer Risk Calculator (ERSPC, www.uroweb.org), a prostate cancer risk assessment tool, is being considered. The diagnostic/prognostic value of this test, introduced early in 2010, needs to be studied in comparison to PCA3.

5.5.18.23 Prostasomes in Blood as Biomarker of Prostate Cancer

Prostasomes are microvesicles (mean diameter, 150 nm) that are produced and secreted by normal as well as malignant prostate acinar cells. In prostate cancer, rather than ending up in semen, prostasomes are pumped out into the surrounding tissue in invasive cancer. Therefore, prostasomes levels are expected to be elevated in blood in patients with prostate cancer and correlate more closely with the severity of the disease than do PSA levels. Prostasomes can be detected by proximity ligation for effective determination of proteins. In this method, the target is first captured via an immobilized antibody and subsequently detected by using four other antibodies with attached DNA strands. The requirement for coincident binding by five antibodies to generate an amplifiable reporter results in both increased specificity and sensitivity. The assay successfully detected significantly elevated levels of prostasomes in blood samples from patients with prostate cancer before radical prostatectomy, compared with controls and men with benign biopsy results (Tavoosidana et al. 2011). This approach that enables detection of prostasomes in peripheral blood may be useful for early diagnosis and assessment of prognosis in organ-confined prostate cancer.

5.5.18.24 PSMA as Biomarker of Prostate Cancer

Prostate PSMA in prostate tissue is increased in patients with more aggressive features, i.e., higher Gleason grade and higher stage. Significant upregulation of PSMA expression occurs in patients with metastatic disease as compared to those with localized prostate cancer and in localized disease compared to benign prostate tissue. High PSMA levels in primary prostate cancer not only correlate with other adverse traditional prognostic factors but also can independently predict both a higher incidence and shorter time to disease recurrence.

Increased PSMA expression in response to treatment with antiandrogen drugs such as MDV3100 and abiraterone can be quantitatively measured in vivo in human prostate cancer xenograft models through PET imaging with a fully humanized,

radiolabeled antibody to PSMA, 64Cu-J591 (Evans et al. 2011). This could serve as a biomarker of AR signaling to noninvasively evaluate AR activity in patients with castration-resistant prostate cancer.

5.5.18.25 Sarcosine as a Metabolic Biomarker of Prostate Cancer

Sarcosine, an N-methyl derivative of the amino acid glycine, has been identified as a differential metabolite that is elevated during prostate cancer progression to metastasis and can be detected noninvasively in urine (Sreekumar et al. 2009). Sarcosine levels are also increased in invasive prostate cancer cell lines relative to benign prostate epithelial cells. Knockdown of glycine-*N*-methyl transferase, the enzyme that generates sarcosine from glycine, attenuates prostate cancer invasion. Addition of exogenous sarcosine or knockdown of the enzyme that leads to sarcosine degradation, sarcosine dehydrogenase, induced an invasive phenotype in benign prostate epithelial cells. AR and the ERG gene fusion product coordinately regulate components of the sarcosine pathway. Profiling of the metabolomic alterations of prostate cancer progression has revealed sarcosine as a potentially important metabolic intermediary of cancer cell invasion.

5.5.18.26 Silenced CDH13 Gene as a Biomarker of Cancer

Biochemical (PSA) recurrence of prostate cancer after radical prostatectomy remains a major problem. Better biomarkers are needed to identify high-risk patients. A MSP assay has been used to assess the methylation state of 15 genes known to influence prostate cancer in prostate cancer tissue samples taken from patients during surgery to remove all or part of the prostate gland (Alumkal et al. 2008). Prostate cancer recurrence, which occurred in one-third of patients within 5 years of their surgery, was linked to silencing of one of these genes, CDH13, which codes the protein cadherin 13 (plays a role in cell–cell adhesion). The results of this study showed that methylation of CDH13 is independently associated with an increased risk of biochemical recurrence after radical prostatectomy even considering the weighted risk of recurrence score. These findings should be validated in an independent, larger cohort of patients with prostate cancer who have undergone radical prostatectomy.

5.5.18.27 Serum Protein Fingerprinting

Protein fingerprinting can be used on serum samples to help accurately distinguish between cancer of the prostate, benign prostate hyperplasia (BPH), and healthy tissue. Proteins are detected by a Protein-Chip array, and an artificial intelligence learning algorithm is used to reduce the number of proteins found down to the number that are required to differentiate prostate cancer from noncancer cohorts. 2D GE

and MS have been used to study serum proteins expressed in patients with BPH and those with high-grade prostatic intraepithelial neoplasm (Gu et al. 2008). Serum amyloid A was found to be expressed in the cancer patients, but weakly or not at all in those with BPH.

New biomarkers, such as autoantibody signatures, may improve the early detection of prostate cancer. With a phage display library derived from prostate cancer tissue, phage protein microarrays were used to analyze serum samples from patients with prostate cancer (Wang et al. 2005b). A panel of peptides performed better than PSA in distinguishing between the group with prostate cancer and the control group. Autoantibodies against peptides derived from prostate cancer tissue could be used as the basis for a screening test for prostate cancer.

Serum-fingerprinting method has a higher specificity than PSA test for differentiating prostate cancer from BPH and unaffected healthy men. This approach can substantially reduce unnecessary prostate biopsies. Another advantage of this technique is that prostate cancer might be detected earlier than with PSA screening. The next step is to identify other biomarkers that can differentiate aggressive cancers from nonaggressive cancers, to make this classification system for early detection as effective as possible.

5.5.18.28 Tests for Prostate Cancer Based on Genetic Dislocations

Gen-Probe has licensed rights from the University of Michigan (Ann Arbor, MI) to genetic translocations that have been shown to be highly specific for prostate cancer in order to develop diagnostic tests for this cancer. Approximately 60–80 % of cancerous prostate tissue contains the translocations, which are not found in healthy prostate tissue. The specific translocations are fusions between prostate-specific, androgen-responsive gene, TMPRSS2, and members of the ETS family of genes, including ERG and ETV1, which were previously known to be involved in other types of cancer (Tomlins et al. 2005). Use of FISH demonstrated that 23 of 29 prostate cancer samples harbor rearrangements in ERG or ETV1. Common genetic translocations have been studied in hematological malignancies such as leukemia, resulting in new molecular tests and therapies. TMPRSS2 is turned on in the presence of androgen. However, this discovery represents the first demonstration of chromosomal rearrangements in a solid tumor such as prostate cancer, where the malignant transformation originates in epithelial cells lining the prostate glands.

5.5.18.29 Concluding Remarks on Biomarkers of Prostate Cancer

Despite many promising candidates, no single biomarker has satisfied the criteria as the ideal biomarker. Limited clinical use of IL-6, TGF-β1, and PCA3 has started, and further widespread availability of these tests is expected in the near future.

The trend is to use artificial neural networks and panels of biomarkers instead of individual assays. Although PSA has some well-known limitations, it remains the best biomarker available for prostate cancer when used in conjunction with nomograms or risk calculators (Martinez et al. 2009).

There is a tremendous need for better prognostic biomarkers in prostate cancer to assist in the identification of patients with aggressive forms of the disease who can potentially benefit from earlier and more intensive forms of treatment. Potential biomarkers of prostatic cancer include caveolin-1, p-Akt, p27, the met oncogene, Ki67 (MIB-1), 8q24 overexpression, Polycomb protein EZH2, plasma TGF-B1, and IL-6, among others.

5.5.19 Renal Cancer Biomarkers

RCC is a form of kidney cancer that involves malignant transformation of cells of the renal tube. It is the most common type of kidney cancer in adults. RCC accounts for approximately 3 % of adult malignancies and 90–95 % of neoplasms arising from the kidney. In the USA more than 32,000 new cases of RCC are diagnosed every year, and approximately 12,000 people die from the disease annually. RCC metastasizes easily, often spreading to the lungs and other organs. In cases where metastatic disease is not yet present at time of diagnosis, the 5-year survival rate for RCC patients is approximately 60–75 %. However, metastases are already present at diagnosis in approximately one-third of RCC cases. In cases where the tumor has metastasized to the lymph nodes, the 5-year survival rate is reduced to 5–15 %. In cases where the cancer has spread to other organs, the 5-year survival rate is less than 5 %.

5.5.19.1 Gene Expression Profile of RCC for Biomarkers

The WHO system defines histopathological tumor subtypes of RCC with distinct clinical behavior and underlying genetic mutations. In adults, the common malignant subtypes are variants of RCC. RCC has a poor prognosis and unpredictable course and to date there are no molecular markers that reliably predict RCC outcome. Histopathological classification of RCC is critical for clinical management of RCC but is becoming more complex with recognition of novel tumor subtypes, development of procedures yielding small diagnostic biopsies, and emergence of molecular therapies directed at tumor gene activity. Therefore, classification systems based on gene expression are likely to become essential for diagnosis, prognosis, and treatment of kidney tumors. Recent DNA microarray studies have shown that clinically relevant renal tumor subtypes are characterized by distinct gene expression profiles, which are useful for discovery of novel diagnostic and prognostic biomarkers (Yin-Goen et al. 2006).

5.5.19.2 miRNA Biomarkers of Renal Cancer

miRNA microarray analysis for comparison of miRNA expression levels between RCC tissues and their normal counterpart revealed several dysregulated miRNAs, which were validated by quantitative RT-PCR and bioinformatic analysis (Chow et al. 2010). Some of these miRNAs are dysregulated in other malignancies as well and have a potential role in RCC pathogenesis. The miRNAs showed a significant correlation with reported chromosomal aberration sites. Target prediction algorithms were to identify gene targets. These miRNAs are potential biomarkers of RCC. A study has demonstrated the usefulness of miRNA expression profiling for identifying a signature unique to various RCC subtypes at a single anatomical locus (Petillo et al. 2009).

5.5.19.3 Use of Proteomics for Detection of RCC Biomarkers

Quantitative MS analysis has been used to identify proteins that are dysregulated in RCC (Siu et al. 2009). Protein expression of kidney cancer tissues was compared to their normal counterparts from the same patient using LC–MS/MS. iTRAQ labeling that enabled simultaneous quantitative analysis. These dysregulated proteins in RCC were statistically significantly different from those of transitional cell carcinoma and end-stage glomerulonephritis. These results were validated using different tools and databases including Serial Analysis of Gene Expression (SAGE), UniGene EST ProfileViewer, Cancer Genome Anatomy Project, and Gene Ontology consortium analysis.

Multivariate analyses using proteomic data obtained by SELDI-MS have been reported to be highly successful for detection of various tumors by examination of serum samples. Scientists at National Cancer Center Research Institute (Tokyo, Japan) have generated proteomic spectra by TOF MS from a set of samples from RCC patients and controls. The simultaneous recognition of two biomarkers was shown to have sensitivity of 89.5 % and specificity 95.0 % for diagnosis RCC and to allow detection of RCC in stage I in 88.9 % of the cases (Hara et al. 2005).

Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) has been identified as a protein that is released from mitochondria in response to apoptotic stimuli and promotes apoptosis by antagonizing inhibitor of apoptosis proteins. Detection and quantification of Smac/DIABLO activity (i.e., protein transcription and expression) in cancerous tissue following biopsy or surgery may be accomplished through IHC, RT-PCR, Western blot analysis, flow cytometry, and/or HPLC.

5.5.19.4 Use of RCC Biomarkers for Prognosis and Therapy

RCC responds poorly to chemotherapy and radiation therapy. Cytotoxic chemotherapy, an integral therapeutic component for solid and non-solid tumors, shows

little or no antitumor activity against RCC. Complete eradication of RCC by chemotherapy is therefore unlikely, unless all of the cancer can be removed by surgery. Radiation therapy is usually unsuccessful in treating RCC and is therefore not commonly used. In light of the fact that chemotherapy and radiation therapy mediate their antitumor effects by inducing apoptosis, poor response of RCC to these conventional therapies has been hypothesized to associate with the signal transduction pathway that mediates apoptosis in RCC. Two key pathways have recently gained particular attention: the hypoxia response pathway associated with the von Hippel–Lindau (VHL) tumor suppressor gene and mTOR signaling pathway. RCC is associated with loss of functional VHL protein (pVHL) and high, homogeneous expression of the G250MN protein, with a close correlation between hypoxia-inducible function (HIF)-1 expression and G250MN. Inherited mutation of the VHL gene is a strong risk factor for RCC and somatic mutation of VHL gene is one of the most frequent genetic changes seen in RCC.

A novel kidney cancer biomarker, transmembrane carbonic anhydrase IX (CAIX), has been investigated as an independent prognostic factor for survival for patients with metastatic RCC. CAIX plays an essential role in allowing cancer cells to buffer their intracellular pH in hypoxia and/or in intensive glycolysis conditions. CAIX proteins may have a role in the regulation of cell proliferation in response to HIF and may be involved in tumor progression. In patients with nonmetastatic RCC, low CAIX predicts a worse outcome similar to patients with metastatic disease and overall CAIX expression decreases with development of metastasis. CAIX reflects significant changes in tumor biology, which may be used to predict clinical outcome and identify high-risk patients for adjuvant-targeted therapies (Said 2005). So far the overall evidence points to a connection between VHL mutation and CAIX expression that can be used as a prognostic marker to predict treatment outcome in patients with RCC. Further studies are needed to determine the molecular pathways involved in such tumors as this would have important therapeutic implications (Patard 2008).

Researchers at UCLA (Los Angeles, CA) have shown that expression level, transcription regulation, and biological activity of Smac/DIABLO determine in large part the pathogenesis of RCC and the response of RCC to antitumor therapies and agents, including chemotherapy, irradiation, immunotherapy, gene therapy, toxins, and antibodies. Smac/DIABLO offers promising utility as a prognostic biomarker for RCC. Smac/DIABLO expression is downregulated in RCC, and lack of Smac/DIABLO expression in RCC predicts a worse prognosis (Mizutani et al. 2005). In addition, transfection with Smac/DIABLO sensitizes RCC to TRAIL/cisplatin-induced apoptosis. These results suggest that Smac/DIABLO expression in RCC may be used as a prognostic parameter and that enhancement of Smac/DIABLO expression in RCC may potentiate immunotherapy and chemotherapy. Analysis of Smac/DIABLO expression will be of significant clinical importance in the design of therapeutic strategies. Smac/DIABLO expression can be induced by use of agents that sensitize cells to apoptosis (e.g., chemicals, inhibitors, biologicals, antisense RNA, gene transfection reagents). An alternative strategy would be to use protease inhibitors to prevent degradation of Smac/DIABLO. Enhanced expression or

reduced degradation resulting from these therapeutic approaches may cause the spontaneous induction of apoptosis or may be used synergistically in combination with low-dose chemotherapy, radiation, or immunotherapy.

5.5.20 Thyroid Cancer Biomarkers

In many cases of papillary thyroid carcinoma (PTC), preoperative diagnoses by fine needle biopsy (FNB) are inconclusive. Although fine needle aspiration cytology is very useful in the diagnosis of PTC, its accuracy and utility would be greatly facilitated by the development of specific biomarkers for PTC and its common variants. Prognostic value of plasma calcitonin and CEA doubling time and the presence of somatic RET mutations in medullary thyroid carcinoma (MTC) tissue may be useful tools in clinical decision-making (van Veelen et al. 2009).

5.5.20.1 Detection of BRAF Mutation

Many effective methods are available to detect BRAF mutation in FNB material. Because of its high specificity, this genetic alteration is now considered a useful diagnostic marker for patients who have indeterminate thyroid nodule cytology and is a useful tool for thyroid nodule management despite its low sensitivity limiting its application. In future, the screening of genetic alterations will enter standard clinical practice as an adjunctive tool to conventional cytology, and larger studies will provide a better definition of the best, most cost-effective combinations of markers and methods (Marotta et al. 2011).

5.5.20.2 Gene Expression Biomarkers of Thyroid Cancer

A comprehensive meta-review of thyroid cancer biomarkers from 21 published studies which uses a gene ranking system that considers the number of comparisons in agreement, total number of samples, average fold-change, and direction of change was devised (Griffith et al. 2006). This approach represents a useful method for identifying consistent gene expression biomarkers when raw data are unavailable. A review of the top 12 candidates revealed well-known thyroid cancer biomarkers such as MET, TFF3, SERPINA1, TIMP1, FN1, and TPO as well as relatively novel or uncharacterized genes such as TGFA, QPCT, CRABP1, FCGBP, EPS8, and PROS1. These candidates should help to develop a panel of biomarkers with sufficient sensitivity and specificity for the diagnosis of thyroid tumors in a clinical setting.

An initial microarray study using the Life Technologies Corp. 1700 Chemiluminescent Microarray Analyzer was performed on surgically resected thyroid lesions to identify molecular signatures that distinguish PTC from benign tissue and to discriminate between common variants of PTC (Finn et al. 2007).

Selected targets were validated using TaqMan® Real-Time PCR. The data generated corroborate such previously identified potential biomarkers as LGALS3, S100A11, LYN, BAX, and CD44. However, the study highlighted numerous transcripts never previously implicated in thyroid carcinogenesis (many of which are not represented on other microarray platforms). Diminished expression of metallothioneins featured strongly, suggesting a possible role as PTC tumor suppressors. Fifteen transcripts were significantly associated with Follicular Variant PTC. The genes in this subcategory were associated with a narrow repertoire of functions, including the MHC and cathepsin families.

5.5.20.3 Multiple Endocrine Neoplasia Type 2B as Risk Factor for Thyroid Cancer

Multiple endocrine neoplasia type 2B (MEN2B) is an autosomal dominant, inherited cancer syndrome. MEN2B patients have a high risk of developing MTC, and prophylactic thyroidectomy is recommended by 6 months of age. Genetic testing can identify MEN2B patients before cancer progression. Two RET proto-oncogene mutations, in exon 15 at codon 883 and in exon 16 at codon 918, account for more than 98 % of MEN2B cases. An assay using unlabeled probes and the LightCycler 480 instrument was developed to genotype these two common MEN2B RET mutations (Margraf et al. 2008). This is a rapid, closed-tube method that is less time-consuming and less expensive than sequencing. This assay demonstrates 100 % specificity and sensitivity for the identification of RET mutations causative of MEN2B.

5.5.20.4 miRNA Biomarkers of Thyroid Cancer

miRNA expression profiling correlates with various cancers, with these genes thought to act as both tumor suppressors and oncogenes. ret/PTC 1 is an oncogene with constitutive kinase activity implicated in the development of PTC. This rearrangement leads to aberrant MAPK activation that is implicated in PTC tumorigenesis.

A cell line matrix model containing normal thyroid, PTC with native BRAF mutation or ret/PTC activation, and normal thyroid cells transfected with either BRAFmut or ret/PTC has been used to investigate transcription and posttranscriptional regulation in PTC using DNA microarray and miRNA analysis (Cahill et al. 2006a). Ambion's RecoverAll™ Total Nucleic Acid Isolation Kit was used to extract RNA for expression microarray and miRNA analysis. The Life Technologies Corp.'s TaqMan® Human Early Release Panel containing 160 microRNA assays designed to detect and quantify mature miRNAs was used to analyze the cell line panel. Distinct gene signatures were found contingent upon the primary genetic mutation (ret/PTC or BRAFmut). A unique miRNA expression signature differentiated between PTC cell lines with ret/PTC, BRAFmut, and a normal thyroid cell line. As miRNAs are stable, abundant, and very easily detectable, they may be ideal candidates for

diagnostic biomarkers. They are also resilient and detectable in archival material. Future exploration on a larger cohort of samples will hopefully consolidate the correlation between these miRNAs and their host genes in PTC and help identify miRNA biomarkers.

5.5.20.5 Biochemical Biomarkers of Thyroid Cancer

MTC shares biochemical features with other neuroendocrine tumors, but some particular characteristics could be used as biomarkers. A study of the biochemical neuroendocrine profile of histologically proven MTC showed that plasma dopamine was increased in the majority of the patients with stable disease and progressive disease, but it did not correlate with extent of disease (Groot et al. 2006). Elevated plasma platelet levels of serotonin were only present in patients with MEN2 with stable disease or progressive disease but did not differ between those groups. Histamine metabolites were elevated in 20 % of patients with stable disease and progressive disease. In addition to plasma calcitonin, only carcinoembryonic antigen (CEA) and CGA could differentiate between stable and progressive MTC. MTCs are capable of synthesizing catecholamines, serotonin, and histamine metabolites indicating metabolic characteristics in common with other neuroendocrine tumors, but clinical usefulness and relevance seems limited. It is concluded that the most useful markers in the follow-up of MTC are plasma calcitonin and CEA.

5.6 Validation of Biomarkers

NCI's EDRN developed a five-phase approach to systematic discovery and evaluation of biomarkers. Biomarker development should follow an orderly process wherein one proceeds to the next phase only after meeting pre-specified criteria for the current phase. Various phases are as follows (Manne et al. 2005):

- Phase I refers to preclinical exploratory studies and is usually characterized by ranking and selection or finding suitable ways to combine biomarkers.
- Phase II has two important components: (1) upon successful completion of the phase I requirements, an assay is established with a clear intended clinical use, and (2) the assay is evaluated for its clinical performance in terms of "sensitivity" and "specificity" with thresholds determined by the intended clinical use.
- Phase III involves evaluation of the sensitivity and specificity of the test for the detection of diseases that have yet to be detected clinically. The specimens analyzed in this evaluation phase are taken from study subjects before the onset of clinical symptoms, with active follow-up to ascertain disease occurrence. Most biomarker validation studies end with this phase, and the biomarker will be ready for clinical use.

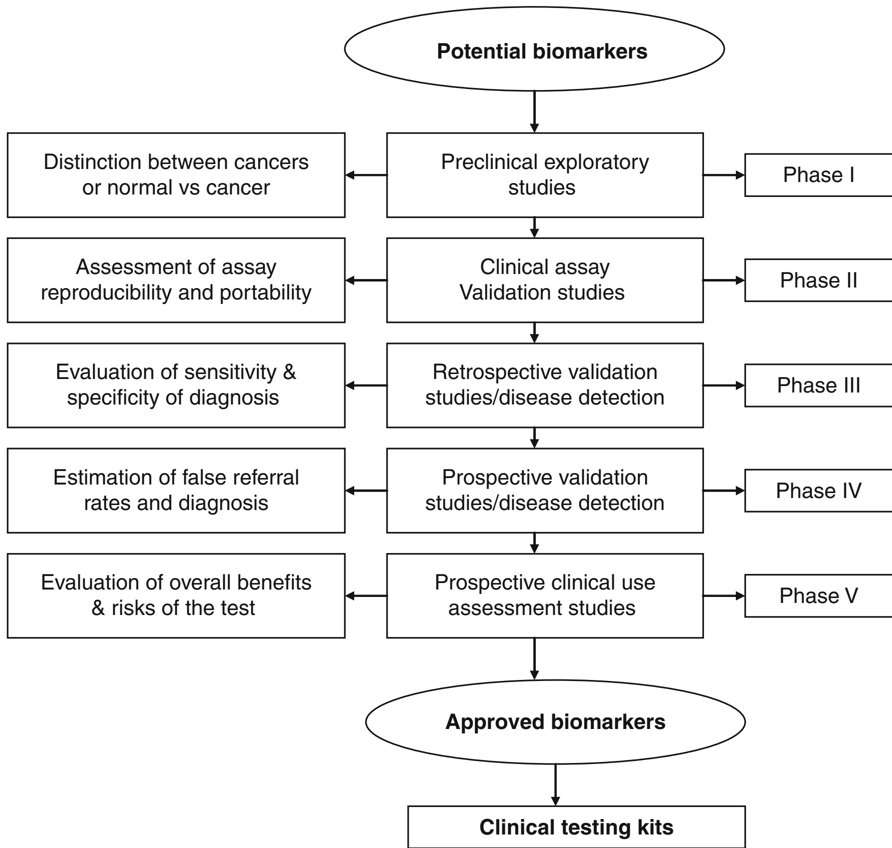

Fig. 5.2 Cancer biomarker development and validation. *Source*: NCI, US Government

- Phase IV evaluates the sensitivity and specificity of the test on a prospective cohort. It can estimate the false referral rate based on tested biomarkers and describe the extent and characteristic of disease detected, e.g., tumor stage at the time of detection.
- Phase V evaluates the overall risks/benefits of the new diagnostic test on the screened population. The cost per life saved is one example of an endpoint for such study.

PSA is an example of a test that did not go through phases IV and V before its widespread clinical use for prostate cancer screening and its clinical benefits are still under debate. Validation of biomarkers for cancer based on NCI-EDRN criteria is shown in Fig. 5.2.

5.7 Future Prospects and Challenges for Cancer Biomarkers

Although there is intense activity toward identifying novel biomarkers for cancers, especially those for early detection, it is not clear whether we already have too many biomarkers described in the literature. Because there is no central repository of data pertaining to any cancer, it is difficult to estimate if we have too many proteins described as potential biomarkers for any cancer. Technical advances in genomics, transcriptomics, and proteomics have facilitated high-throughput studies in which the data are analyzed in isolation, and a comparison with the published literature is not generally possible for the entire dataset. A central repository will not only integrate all the information scattered across the literature but will also serve as a reference for prioritizing and systematic testing of candidate biomarkers.

Out of the thousands of biomarkers for cancer that have been discovered, <20 are approved by the FDA. With the advent of new and improved genomic and proteomic technologies such as DNA and tissue microarray, 2D GE, MS, and protein assays coupled with advanced bioinformatic tools, it is possible to develop biomarkers that are able to reliably and accurately predict outcomes during cancer management and treatment. In years to come, a serum or urine test for every phase of cancer may drive clinical decision-making, supplementing or replacing currently existing invasive techniques. A major challenge will be the integration of proteomics with genomic and metabolomic data and their functional interpretation in conjunction with clinical results and epidemiology. A number of genes are up- and downregulated in cancer, making it problematic to rely on any single tumor biomarker even for one type of cancer, whereas the physiological properties of the microenvironment of a majority (90 %) of tumors, such as hypoxia, acidity, and changes in temperature, are considered promising environmental markers for tumor targeting. Hypoxia and acidosis are hallmarks of tumors at both very early and advanced stages of tumor development.

Up to now considerable scientific effort has gone into finding common SNPs that correlate to risk association for cancer. Protein biomarkers are now considered to be more effective in risk assessment, early detection, and cancer prevention. The discovery phase for biomarkers should be ramped up and multiplexed. Whereas traditional drug discovery is limited to putting just one therapeutic in a patient or model organism at a time, a single serum or tissue sample from a patient can serve as a test for thousands of biomarkers at once. Multiplexing in this way could make trials much more high throughput and accelerate protein biomarker discovery.

Targeted therapeutics has provided challenges for imaging techniques to assess tumor response to treatment because many new agents cause cytostasis rather than cytotoxicity. Advanced tracer development, image acquisition, and image analysis have been used to produce quantitative biomarkers of pathophysiology, with particular focus on measurement of tumor vascular characteristics. A critical review of the available technologies leads to the conclusion that there is a need for developing comprehensive compound-specific imaging biomarkers that are appropriate for

every class of targeted therapeutics and for investigation of the complementary information given in multimodality imaging studies of targeted therapeutics (O'Connor et al. 2007).

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Chapter 6
Molecular Diagnosis of Cancer

6.1 Introduction

Cancer is a multifactorial disease involving interaction of genetic, environmental, hormonal, and dietary risks. Genes play an important role. Oncogenomics (Chap. 2), sequencing (Chap. 3), oncoproteomics (Chap. 4), and biomarkers (Chap. 5) are relevant to molecular diagnosis of cancer. Basic molecular diagnostic technologies are described in detail in a special report on this topic (Jain 2013). Some of the technologies used for diagnosis of cancer are described in this chapter.

6.1.1 CNVs in Cancer

The cancer genome is moulded by the dual processes of somatic mutation and selection. Homozygous deletions in cancer genomes occur over recessive cancer genes, where they can confer selective growth advantage, and over fragile sites, where they are thought to reflect an increased local rate of DNA breakage. However, most homozygous deletions in cancer genomes are unexplained. Microarray-based experiments and targeted sequencing to identify signatures of selection and mutation in ~750 cancer cell lines have identified 2,428 somatic homozygous deletions (Bignell et al. 2010). These overlie 11 % of protein-coding genes that, therefore, are not mandatory for survival of human cells. The authors derived structural signatures that distinguish between homozygous deletions over recessive cancer genes and fragile sites. Application to clusters of unexplained homozygous deletions suggests that many are in regions of inherent fragility, whereas a small subset overlies recessive cancer genes. The results illustrate how structural signatures can be used to distinguish between the influences of mutation and selection in cancer genomes. The extensive copy number, genotyping, sequence, and expression data available for this large series of publicly available cancer cell lines render them informative reagents for future studies of cancer biology and drug discovery.

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_6,
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Another powerful way to discover key genes with causal roles in oncogenesis is to identify genomic regions that undergo frequent alteration in human cancers. Copy number analyses have been used to gain insights into the genomic features of cancer. A high-resolution analysis of somatic copy number alterations (SCNAs) from 3,131 cancer specimens, belonging largely to 26 histological types, has identified 158 regions of focal SCNA that are altered at significant frequency across several cancer types (Beroukhim et al. 2010). Of the 158 regions, 122 cannot be explained by the presence of a known cancer target gene located within these regions. Several gene families are enriched among these regions of focal SCNA, including the bcl2 family of apoptosis regulators and the NF-κB pathway. Comparison of copy number profiles in the cancer samples with those in nearly 1,500 normal tissue samples revealed 75,500 gains and 55,101 losses in the cancer genomes. It was shown that cancer cells containing amplifications surrounding the MCL1 and bcl2L1 antiapoptotic genes depend on the expression of these genes for survival. It was also demonstrated that a large majority of SCNAs identified in individual cancer types are present in several cancer types. Because numerous copy number alterations overlap between different cancer types, it may eventually be possible to glean insights from the pattern of genomic alterations in a tumor rather than just where it originates in the body. Copy number patterns may also provide clues about how to treat cancers. With the ongoing advances in genome technology, it will become possible to decode the genomes of thousands of cancers to reveal every genomic change.

6.1.1.1 Allele-Specific Copy Number Analysis of Tumors

Allele-specific copy number analysis of tumors (ASCAT) is a bioinformatic approach to accurately dissect the allele-specific copy number of solid tumors, simultaneously estimating and adjusting for both tumor ploidy and nonaberrant cell admixture (Van Loo et al. 2010). This allows calculation of "ASCAT profiles" (genome-wide allele-specific copy number profiles) from which gains, losses, copy number-neutral events, and loss of heterozygosity (LOH) can accurately be determined. In an early-stage breast carcinoma series, aneuploidy was observed in 45 % of the cases and an average nonaberrant cell admixture of 49 %. By aggregation of ASCAT profiles across our series, the authors of this method obtained genomic frequency distributions of gains and losses, as well as genome-wide views of LOH and copy number-neutral events in breast cancer. In addition, the ASCAT profiles reveal differences in aberrant tumor cell fraction, ploidy, gains, losses, LOH, and copy number-neutral events between the five previously identified molecular breast cancer subtypes. Basal-like breast carcinomas have a significantly higher frequency of LOH compared with other subtypes, and their ASCAT profiles show large-scale loss of genomic material during tumor development, followed by a whole-genome duplication, resulting in near-triploid genomes. Finally, from the ASCAT profiles, a genome-wide map of allelic skewness in breast cancer was constructed, indicating loci where one allele is preferentially lost, whereas the other allele is preferentially gained.

6.1.2 *Viruses and Cancer*

Approximately 20 % of human cancers have a viral etiology. The incidence of such cancers, however, is lower than the frequency of the viral infections; thus, mechanisms other than the presence of virus play a role in the initiation of these oncological events. Transforming retroviruses carry oncogenes derived from cellular genes that are involved in mitogenic signaling and growth control. DNA tumor viruses encode oncogenes of viral origin that are essential for viral replication and cell transformation; viral oncoproteins complex with cellular proteins to stimulate cell cycle progression and led to the discovery of tumor suppressors. Improvements in the detection of viruses and biomarkers of chronic infection have led to the identification of strong associations with cancer, particularly for human papillomavirus (HPV), hepatitis B virus (HBV), and human immunodeficiency virus (HIV). Detection of viruses by molecular diagnostic examination of the tumor tissue can illuminate the pathogenesis of some of the tumors. Some viruses encode proteins that can subvert the function of host-cell-encoded tumor suppressor genes. In turn, gene therapy approaches may be able to restore the proper function. Selected viruses that are linked to human cancer are listed in Table 6.1.

Table 6.1 Viruses linked to human cancer

Virus	Cancer	Possible mechanism
Human T-cell leukemia virus (HTCLV-1)	Adult T-cell leukemia (ATL)	HTLV-1 encodes a putative oncogene, *Tax*, which deregulates growth-related genes
Herpes-like viruses	Kaposi's sarcoma Lymphomas in AIDS	Herpes virus-like DNA sequences found in lesions Microsatellite instability
Human papilloma virus (HPV)	Cervical cancer	Epidemiological evidence. HPV-encoded proteins E6 and E7 antagonize the apoptotic response of cells to DNA damage
Epstein–Barr virus (EBV)	1. Immunoblastic lymphoma	1. Long-term immunosuppression
	2. Nasopharyngeal carcinoma	2. Expression by abnormal cells of EBV-encoded latent membrane protein which is essential for the transformation of B lymphocytes into lymphoblastic cell lines
Hepatitis B virus (HBV)	Hepatocellular carcinoma (HCC)	HBVs have a 200-fold higher risk of developing HCC. Incorporation of the gene for HBVx protein into the genome alters the expression of growth regulatory genes. Loss of heterozygosity (LOH) at chromosome loci 11p and 13q (regions containing tumor suppressor genes)
Kaposi's sarcoma herpesvirus (KSHV)	Kaposi's sarcoma in AIDS	Loss of immune function in AIDS

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6.1.2.1 Detecting Viral Agents in Cancer

Identifying a causal viral agent helps in understanding the biology of cancers induced by viruses and can lead ultimately to the development of antiviral drugs and vaccines for their treatment and prevention (Dalton-Griffin and Kellam 2009). The ability of some agents to remain latent, as well as the existence of new and emerging viral infections, provides challenges for detection of cancer and proving its cause. A virus could trigger the initial events of oncogenesis but may be absent in the final tumor, which adds timing of detection to the problem. However, once the causal agent has been unequivocally found, development of a preventive treatment can be relatively rapid, as has been the case for cervical cancer. HPVs, especially types HPV16 and HPV18, are now firmly associated with cervical cancer; this led to the development and widespread use of a HPV vaccine. Similarly, recovery of immune function in AIDS by inhibiting HIV replication by antiretroviral therapy can lead to a regression of Kaposi's sarcoma, an endothelial tumor caused by the Kaposi's sarcoma herpesvirus (KSHV), and a decrease in incidence of other AIDS-related cancers.

Treating viral infection is therefore a valuable addition to antitumor therapy if the infectious agent can be identified. Currently, two main techniques are used to detect viral genomes in disease, one based on hunting for acknowledged candidates and the other on removing (either physically or computationally) known human sequences to reveal any foreign nucleic acid. PCR and microarray-based strategies are limited by a finite number of probes and sequences available but can be very sensitive.

A subtractive method known as digital transcript subtraction (DTS) attempts to identify exogenous pathogen transcripts via high-throughput sequencing and can thus potentially identify the presence of RNA and DNA viruses. This method involves developing a long serial analysis of gene expression (SAGE) library from the tumor cells by quantitatively joining 21-bp tags composed of cDNA copied from the 3′ end of mRNAs. It can therefore detect all transcripts that are expressed in the tumor. As all human tumor viruses to date express part of their genome in the transformed cells, this has proved effective in virus discovery. DTS has been validated by identifying sequences from KSHV in the primary effusion lymphoma cell line BCBL-1 (Feng et al. 2007). More recently, DTS has been used to identify a new polyomavirus in an uncommon but aggressive human skin cancer, Merkel cell carcinoma (Feng et al. 2008). A fusion transcript between an unknown virus T antigen and a human receptor tyrosine kinase was detected. The new virus was named Merkel cell polyomavirus (MCV) and was detected in 80 % of MCC tumors and also in 16 % of normal skin biopsies. In 75 % of the MCV-related MCCs, viral DNA was integrated in a clonal pattern, suggesting a potential mechanism for transformation. Merkel cell carcinoma occurs predominantly in the elderly and immunosuppressed, two of the key features that indicate an infectious etiological agent.

DK-MICROBE applies computational subtraction to digital karyotyping to hunt for virus genomes in several primary colorectal cancer (CRC) samples and metastases as well as in normal tissue (Duncan et al. 2009). It aims to circumvent

limitations on detection imposed by the different mechanisms by which pathogens contribute to disease. In DK-MICROBE, genomic DNA from the tumor is digested enzymatically into fragments of less than 10 kb in size that are processed to yield 21-bp tags for amplification, concatenation, and sequencing. Human sequences are computationally removed; the remaining unidentified "pathogen" tags are then studied further. However, DK-MICROBE in its present form can only detect the genomes of DNA viruses, e.g., human herpesvirus 6 genome in samples from tumor tissue. The fact that they also identify the viral DNA in healthy tissues indicates the difficulties of causally associating a particular virus with a particular cancer.

6.2 Conventional Cancer Diagnosis

Clinical suspicion of cancer requires confirmation by laboratory diagnostic investigations. The conventional investigations include imaging studies, histopathology and cytological examination, and measurement of antibodies.

Modern radiological imaging techniques can illuminate characteristic features of some malignant tumors. Computerized tomography (CT), for example, represented a great improvement over conventional radiography, particularly in the diagnosis of brain tumors. In addition, CT is approximately 15–20 % more accurate than lung tomography in the diagnosis of pulmonary metastases. Its limitations center on its lack of tissue specificity (which makes tumors below 1.5 cm difficult to detect) and the potential for exposure to radiation.

Magnetic resonance imaging (MRI) overcomes some of the drawbacks of CT through its multiplanar display, good spatial resolution, high tissue contrast, and lack of radiation exposure. This technique is most valuable in detecting tumors of the nervous system, musculoskeletal system, and bone marrow.

Positron emission tomography (PET) has been used in diagnosis of tumors since the 1970s. Its spatial resolution is less than that of MRI or CT, but it is the most sensitive and specific technique for imaging molecular pathways in vivo in man and is developing into an in vivo molecular diagnostic method.

The standard approach in cancer diagnosis involves microscopic examination of a stained section of the tissue (obtained by biopsy or aspiration), with diagnosis being based on the sample's morphology and staining characteristics. Electron microscopy provides a greater level of resolution than light microscopy. One limitation of microscopic evaluation of selected tissue sections is that it is not necessarily representative for the whole biopsy, so areas of diagnostic interest could be missed. Immunohistochemistry (IHC) is of special interest, because it correlates detection of a cancer-specific antigen with visualization of shape and morphology in cells or tissue sections.

Cytological examination of various body fluids and effusions is also performed. In the case of hematological malignancies, analysis can be performed on samples drawn from peripheral blood, bone marrow, and lymph nodes. Cytogenetic analysis, which evaluates the gross morphologic features of chromosomes, is especially

useful in detecting consistent, nonrandom chromosomal abnormalities (e.g., translocations and deletions). Image analysis, which uses a computer to analyze digitalized microscopic images, also aids in DNA ploidy analysis. This technology is complementary to flow cytometry in assessing the DNA content and proliferative capacity of a tumor.

6.3 Molecular Techniques for Cancer Diagnosis

Molecular genetics has been pivotal in expanding our understanding of the biological mechanisms operative in carcinogenesis and delivering helpful tools for cancer diagnosis. These technologies are rapidly gaining in importance as technical adjuvants in a more refined approach to diagnosis and follow-up of particular malignancies. They also represent important tools for the presymptomatic diagnosis of individuals from families afflicted with hereditary conditions that predispose them to particular cancers. Molecular diagnosis is assuming increasing importance for determining prognosis and selecting as well as monitoring treatment in personalized management of cancer. Various approaches to molecular-based diagnostics for cancer are:

1. Identification of novel genes associated with a particular type of cancer. The limitation is that it only provides information on predisposition.
2. The proteomic approach is to identify the most promising protein targets (tumor marker proteins). The limitation of this approach is that promising protein targets are only found in tissue, and not in blood.
3. Antibody-based detection systems are the most widely used for identifying tumor antigens. Although the reliability of these methods has improved with technological advances, the inherent potential for nonspecificity and cross-reactivity in any immunological reaction warrants caution when interpreting these results. Measurement of cancer antibodies in blood has several advantages over other molecular diagnostic technologies, as cancer-specific antibodies are detectable prior to the detection of tumor marker proteins or palpable tumor.

Table 6.2 shows molecular diagnostic techniques applicable to the diagnosis of cancer.

6.3.1 Genome Analysis at the Molecular Level

When carried out at the molecular level, genome analysis can provide high resolution of the tumor DNA and the patient's constitutional DNA, enabling the subsequent detection of allelic imbalances by Southern blotting. This approach uses naturally occurring polymorphisms to obtain the distribution of the recognition sites for specific restriction enzymes (RFLP). Because this analysis utilizes

Table 6.2 A classification of molecular diagnostic methods in cancer

Genomic technologies
Assays for determining genetic susceptibility to cancer
Comparative genomic hybridization
DNA tags for finding genes expressed in cancer
Fluorescent in situ hybridization (FISH)
Genome analysis at the molecular level
Restriction fragment length polymorphism (RFLP)
Loss of heterozygosity (LOH)
Digital karyotyping
Nonradioactive mutation screening
Single-strand conformation polymorphism (SSCP)
Polymerase chain reaction (PCR)
Reverse transcriptase (RT)-PCR
Simultaneous hybridization of an arbitrarily primed (SHARP) PCR
Real-time quantitative PCR
Gene expression profiling
cDNA microarrays to analyze gene expression
Serial analysis of gene expression (SAGE)
MicroRNA expression profiling
Expression profiling of tumor cells sorted by flow cytometry
Detection of molecular markers of cancer
Oncoproteins as markers for cancer
Detection of DNA methylation
LigAmp for detection of gene mutations in cancer
Measurement of serum nucleosomes as indicator of response to chemotherapy
In vivo molecular diagnosis of cancer by positron emission tomography
Detection of tumor cells in body fluids
Detection of tumor cells in circulating blood
Use of caspase inhibitors to stabilize circulating carcinoma cells in blood sample
Detection of tumor cells in urine
Detection of tumor cells in cerebrospinal fluid
Assays based on proteins and enzymes
p53 sequencing and functional assays
Measurement of telomerase activity
Prognostic assay based on survivin (inhibitor of apoptosis protein)
Molecular histopathology of cancer: immunohistochemistry (IHC)
Recombinant antibodies for cancer diagnosis
Proteomic technologies
Aptamer-based technology for protein signatures of cancer cells
Gel electrophoresis
Laser capture microdissection (LCM)
Nanobiotechnology: nanoparticle-based diagnosis combined with therapeutics
Sequencing

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biochemically extracted DNA from fresh or frozen tumor tissue, no information is obtained at the single-cell level. In addition, contamination of the tumor DNA with that from admixed nonneoplastic cells may create interpretational problems. The application of DNA polymorphism to the study of human cancers has, however, provided important clues as to the biological significance of imbalances in chromosomal material, which are frequently observed in cytogenetic studies of tumors. Allelotyping of the individual tumors requires considerable labor and would take several months with the current set of histopathological classification systems.

Gel electrophoresis is a useful method in cancer molecular diagnostics. It is at least ten times as sensitive as Southern blot assay. It can readily determine clonality in the primary diagnosis of a neoplasm. Comparative genomic hybridization (CGH) can accurately identify multiple or missing DNA sequences along the entire chromosome with the aid of biochips.

6.3.2 Mutation Detection at the Molecular Level

It is known that genetic mutations are responsible for sensitizing some tumor cells to chemotherapy, while other mutations render tumor cells completely resistant to drug treatments. Research progress in this area has been slow because analysis of DNA from tumors is complicated by varying amounts of tumor cells in patient samples. Furthermore, the heterogeneous nature of many tumors makes it difficult to accurately sequence the tumor DNA, which is required in order to personalize treatment. This is compounded by cost-prohibitive, conventional low-resolution sequencing methods that lack sufficient accuracy to characterize the DNA in cancerous cells.

6.3.3 Expression Profiling of Tumor Cells Sorted by Flow Cytometry

Microarray technologies have made possible comprehensive analyses of nucleic acid sequence and expression. However, the technology to obtain efficiently high-quality RNA and DNA suitable for array analysis from purified populations of neoplastic cells from human tissues has not been well addressed. Microdissection can enrich for populations of cells present in various tumor tissues, but it is not easily automated or performed rapidly, and there are tissues in which cells of interest cannot be readily isolated based on morphologic criteria alone. RNA and DNA can be isolated efficiently from flow cytometrically purified whole epithelial cells obtained from primary tissue. The aqueous reagent, RNAlater, which preserves RNA, enables immunolabeling and purification of whole epithelial cells by flow sorting without special instrument preparation to reduce RNase activity. Real-time PCR can be used for determining RNA quality after flow sorting. High-quality RNA and DNA suitable for expression and genotype analysis can be readily obtained from flow cytometrically purified populations of neoplastic cells from human tissues.

6.3.4 MicroRNA Expression Profiling for Cancer Diagnostics

Numerous microRNAs (miRNA) have been associated with cancer. Although their function is not well understood, they control gene activity and play a major role in the development of human cancers. Bead-based flow cytometric expression profiling of miRNA in samples from multiple human cancers has shown distinctive microRNA fingerprints. Generally there was downregulation of miRNAs in tumors compared with normal tissues. The miRNA profiles reflect the developmental lineage and differentiation state of the tumors and enabled successful classification of poorly differentiated tumors, whereas mRNA profiles were highly inaccurate when applied to the same samples. These findings highlight the potential of miRNA profiling in cancer diagnosis and to select the most appropriate treatment. A number of miRNA-based tests are already available for cancer. However, miRNA assays do not currently have an accepted control against which they can be compared, nor have they demonstrated ease in reproducibility. Nevertheless, miRNAs have the potential to compete with commercially available molecular diagnostic assays for cancer for clinical application. These are described in more detail in Chap. 13.

6.3.5 Molecular Fingerprinting of Cancer

Current cancer diagnostics are largely based on either imaging or the analyses of several biochemical markers. However, these methods are not precise, as they do not reveal the molecular structure of the tumor cells and, in most cases, do not allow for early detection. Several companies and research groups are developing proteomic-based diagnostics that identify cancer-related protein and peptide patterns in the blood serum. Although these diagnostics, when fully developed, have the potential to be precise and suitable for early detection, they will not provide the molecular signature or fingerprint of individual tumors. Also under development are molecular diagnostic techniques based on genomic and proteomic analyses of tumor samples. While this method is precise and results in a molecular characterization of cancer cells, it is inadequate for early detection, mass screening, and monitoring because it requires a biopsy from the tumor. Unfortunately for patients, by the time the tumor is detected, it is typically too large and too advanced for effective treatment.

To overcome some of these limitations, a "molecular fingerprinting"-based blood test for cancer has been developed by CeMines Inc. The basis of this test is that antibodies, which reflect the molecular signature or "fingerprint" of a tumor, are generated in cancer's earliest stages, even when the tumor is a collection of single cells. In multisite clinical trials, it correctly identified the cancer with 100 % sensitivity in patients who were known to have one of four different tumor types—lung, breast, gastrointestinal, or prostate. CeMines' blood test also correctly ruled out cancer in control patients with 100 % specificity. Based on these data, CeMines and a research team at Karolinska Institutet (Stockholm, Sweden) have initiated a sponsored research program to validate "molecular fingerprinting" in clinical study in a

large series of cancer patients. The next step will be systematic and well-defined analyses of tumors in order to reach the ultimate goal of "molecular fingerprinting," i.e., deciphering the molecular structure of individual tumors. The advantages of this technology are:

- Early/accurate detection with fewer false positives and false negatives
- Minimally invasive testing of blood or body fluids such as urine, sputum, and saliva
- Prediction as to which of the available treatments is optimal for a specific patient
- Helps to design specific diagnostic methods and drugs to treat cancer
- Increases patients' chances for survival

6.3.6 Fluorescent In Situ Hybridization

DNA in situ hybridization methods permit the investigation of genetic alterations within the context of cell morphology and tissue architecture; such modifications have particular importance in tumor pathology. Fluorescent in situ hybridization (FISH) offers several advantages when applied to tumor diagnosis:

- Chromosomal information can be obtained from nonmitotic lesions.
- Normal cells of the same type present on the same slide serve as internal controls for both the reagents and the hybridization procedure.
- Data can be obtained from very small lesions, whereas conventional metaphase cytogenetic analyses or mutational analysis by Southern blotting or PCR would be limited by dilution of the sample with normal tissue elements.
- Although PCR can illuminate alterations involving fewer cells than FISH can recognize, the exquisite sensitivity of PCR turns into a disadvantage if the clonal aberration that marks the tumor appears in only a small number of cells.
- Rapid (within 24 h) reporting is possible.
- FISH can relate genetic markers to tissue pathology.

6.3.7 Genetic Analysis of Cancer

6.3.7.1 Comparative Genomic Hybridization in Cancer Diagnostics

CGH has been utilized to identify DNA copy number abnormalities in various kinds of cancers, and several reports have shown its usefulness in screening of the genes involved in carcinogenesis and also in the identification of prognostic factors in cancer. CGH has contributed significantly to the current knowledge of genomic alterations in hematological malignancies. Characteristic patterns of genomic imbalances not only have confirmed recent classification schemes in non-Hodgkin's lymphoma, but they provide a basis for the successful identification of genes with

previously unrecognized pathogenic roles in the development of different lymphomas. There are technical limitations, but a procedure termed matrix-CGH overcomes most of the limitations and opens new approaches for diagnostics and identification of genetically defined leukemia and lymphoma subgroups. Current efforts focus on developing leukemia-specific matrix-CGH DNA chips, which are designed to meet the clinical needs.

CGH data are highly reproducible, making this a useful tool in identifying cancer-associated DNA amplifications and, therefore, precancerous lesions. Although the initial results derived from CGH arrays (identification of regions of increased or decreased DNA copy numbers) might not provide complete information about the cellular abnormalities of a tumor, CGH could be followed up by a more detailed analysis. For example, the regions of DNA copy number loss might be further investigated by using LOH analysis or direct sequencing of candidate genes. Similarly, increases in DNA copy number can be further analyzed by in situ hybridization using chromosome-specific or gene-specific probes.

6.3.7.2 Loss of Heterozygosity

The term "loss of heterozygosity" (LOH) is applied to describe the loss of polymorphic DNA markers in tumors compared with normal cells, which often indicates somatic deletion of tumor suppressor genes. This decline has been shown to have prognostic significance in colon cancer. To carry out this analysis, equipment such as the ABI Prism fluorescent technology (Applied Biosystems/Life Technologies) is used. Illumina Inc. has software tools that enable the use of Infinium™ SNP genotyping data to analyze DNA copy number changes and characterize LOH. These two conditions provide informative molecular signposts of cancer development and progression and offer potential to help researchers discover diagnostic and therapeutic targets. Copy number changes occur when a chromosomal sequence or an entire chromosome is amplified erratically or deleted altogether. Such copy number aberrations are typical of cancer cells and may provide clues to help identify genes, promoter regions, and biomarkers implicated in the unchecked growth patterns of cancer cells. LOH indicates cancer onset and/or progression since it can sometimes occur without apparent copy number changes.

6.3.7.3 Digital Karyotyping

Digital karyotyping has been developed for a genome-wide analysis of DNA copy number alterations at high resolution. The principle of this approach is similar to the SAGE method, which is based on the isolation and enumeration of short sequence tags. However, the sequence tags in digital karyotyping are obtained from genomic DNA rather than from mRNA, and they are isolated by different methods. These tags contain sufficient information that allows assigning the tag sequences to their corresponding genomic loci from which they are derived. The number of each

unique tag along each chromosome can be used to quantitatively evaluate DNA content in tumor samples. Digital karyotyping can identify all the known chromosomal alterations and reveal several distinct genetic alterations that are not shown by other methods. Several undiscovered copy number alterations exist in cancer genomes, and many of these could be detected through digital karyotyping. An example is the detection of amplifications in chromosomes 1p34.2 and 17q12 from isolated cells in ovarian carcinoma, which are known to contain L-myc and Her2/neu oncogenes, respectively. Digital karyotyping has also been applied in identifying specific gene amplification in CRC patients that is associated with resistance to chemotherapy with 5-fluorouracil.

6.3.7.4 Gene Expression Profiles Predict Chromosomal Instability in Tumors

Microscopic examination of tumor specimens cannot always predict a cancer's aggressiveness, leading to increased interest in molecular approaches to diagnosis. Now, researchers in the Children's Hospital Informatics Program (CHIP) at the Harvard-MIT Division of Health Sciences and Technology report that a genetic profile indicating chromosomal instability—an increased tendency to develop chromosomal aberrations, critical in cancer development—is predictive of clinical outcome in a broad range of cancer types (Carter et al. 2006).

Chromosomal instability leads to a condition known as aneuploidy, in which chunks of DNA are either missing or duplicated. The technique indirectly measures the degree of aneuploidy and thus the degree of chromosomal instability by looking for abnormal expression levels of genes at the different chromosomal locations. The authors identified a 25-gene signature of chromosomal instability from specific genes whose expression was consistently correlated with total functional aneuploidy in several cancer types. This signature was a significant predictor of clinical outcomes in a variety of cancers (breast, lung, medulloblastoma, glioma, mesothelioma, and lymphoma). It could also differentiate between primary tumors and tumor metastases and in grade 1 and grade 2 breast cancer, distinguished the more aggressive cancers within each grade.

Using data on gene expression (activity) from 18 previous studies of cancer, representing six cancer types, they found that this genetic profile, or signature, predicted poor clinical outcome in 12 of the populations studied. The technique may form the basis of a diagnostic tool that could be used in the clinic and also help in the search for cancer drugs that reduce chromosomal instability.

6.3.8 PCR Techniques

Strategies using PCR can detect clonality with high sensitivity. This type of analysis, which can be performed on DNA from paraffin-embedded tissues, produces results

within a day. When combined with DNA sequencing, PCR can analyze tumor samples rapidly and specifically for mutations related to clonal expansions. Examples of cancer molecular diagnosis situations in which PCR is useful include the following:

- PCR can produce good results even when insufficient material is obtained at biopsy.
- This technology can now detect and even directly sequence p53 gene structural alterations in individual or clustered cells.
- A rapid and sensitive assay based on radiolabeled PCR, followed by restriction enzyme digestion, can differentiate between wild-type and mutant ras genes. This distinction would facilitate analysis of ras gene point mutations in clinical tumor specimens in which ras oncogene activation is an early event in carcinogenesis.
- The analysis of DNA from archival tumor tissues has also been facilitated by the use of PCR.
- K-ras point mutations can be detected by an enriched PCR-colorimetric assay.
- SHARP (simultaneous hybridization of an arbitrarily primed) PCR is a method for detecting mutations in genes for which nucleotide sequences are known. This type of analysis of DNA abnormalities has been applied to samples of human cancers. The use of SHARP can provide information on accumulated genetic abnormalities.

6.3.8.1 COLD-PCR

COLD-PCR (co-amplification at lower denaturation temperature-PCR) is a novel form of PCR that amplifies minority alleles selectively from mixtures of wild-type and mutation-containing sequences irrespective of the mutation type or position on the sequence (Li and Makrigiorgos 2009). For clinically relevant microdeletions, COLD-PCR enabled exclusive amplification and isolation of the mutants. It enriches mutations in cancer samples where normal DNA predominates. The range of enrichment demonstrated to date varies from 3- to 100-fold. Its effectiveness has been demonstrated in enriching for mutations in cancer-related genes in samples where DNA sequencing cannot detect very low concentrations of somatic DNA mutations. The technique was developed at Dana-Farber Cancer Institute (Boston, MA) and has been licensed by Transgenomics for further development and use in combination with its WAVE DHPLC and Surveyor Nuclease products for mutation detection in cancer.

6.3.8.2 Real-Time qPCR for Diagnosis of Cancer

Real-time quantitative PCR (qPCR) is the measurement of a fluorescent signal generated and measured during PCR as a consequence of amplicon synthesis. When used as reverse transcriptase-PCR (RT-PCR), real-time qPCR has proved to be

useful in accurately measuring expression levels of specific gene transcripts. When applied to questions of minimal residual disease (MRD), the technique has evolved from generically detecting the presence of disease cells in individuals, such as the AML1–ETO fusion transcript, to the identification of a specific gene, such as bcl-6, which is prognostic for determining the therapeutic outcome of patients with diffuse large-B-cell lymphoma. Real-time qPCR is used for the study of MRD in leukemia. It reverse-transcribes hybrid bcr–abl mRNA from myelogenous leukemia cell lines and preferentially amplifies cDNA templates from affected cells. Real-time qPCR will play an increasingly important role in clinical testing because it can provide information about gene expression, gene amplification or loss, and small alterations (e.g., point mutations). In addition, it can be applied to detect and quantify viral causes of cancer.

6.3.8.3 Real-Time PCR with myT™ Primer Reagents

myT™ Primer reagents (Swift Biosciences Inc.) have unique structural and thermodynamic properties that enable highly sensitive mismatch discrimination to improve qPCR assays for the detection of key cancer mutations. myT BRAF can detect 1 % mutant BRAF V600E/K present in a background of 103 wild-type genomic DNA copies with no breakthrough amplification from wild type. myT BRAF performs well with either formalin-fixed paraffin-embedded (FFPE) or fresh frozen samples. myT BRAF-Ultra, an ultrasensitive version, provides 0.01 % sensitivity with ability to detect a single copy of mutant template molecule without amplifying any of the approximately 14,000 wild-type copies of genomic DNA over 55 cycles. This is particularly useful where the target is present at very low concentration, such as with circulating tumor cells (CTCs), serum, plasma, and needle biopsies. Swift is also developing a set of myT K-ras primers for high-sensitivity detection of mutations in codons 12 and 13. Vera Diagnostics has licensed myT to incorporate it into its real-time PCR-based molecular diagnostic products that will provide assays with highly sensitive mutation detection capabilities.

6.3.9 Antibody-Based Diagnosis of Cancer

Therapeutic use of MAbs is described in Chap. 8.

6.3.9.1 Monoclonal Antibodies for Diagnosis of Cancer

A monoclonal antibody (MAb) is an antibody made from a single clone (hybridoma) of white blood cells. MAbs were originally designed for therapeutics as described in Chap. 8 and have been used in in vitro cancer diagnostics. There is now a considerable potential for applications of MAbs in in vivo cancer diagnosis. Approved MAb

Table 6.3 Approved monoclonal antibodies for cancer diagnosis

Name/company	Antigen	Format	Mode of action	Indication for use
CEA-Scan/Immunomedics	CEA	Fab conjugate	Radiolabeled with technetium-99m	Colorectal cancer
ProstaScint/Cytogen	PSMA	IgM conjugate	Radiolabeled with indium-111	Detection of extension of prostate cancer
Verluma/NeoRx Corp.	CD20	Fab conjugate	Radiolabeled with technetium-99m	Small-cell lung cancer

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products for cancer diagnosis are shown in Table 6.3. MAbs approved for in vivo diagnosis are still murine although mostly humanized murine or human MAbs are used for therapy.

6.3.9.2 Recombinant Antibodies as a Novel Approach to Cancer Diagnosis

Recombinant antibodies are a serious competition nucleic acid-based diagnostics as these antibodies and their fragments currently represent over 30 % of all biological proteins undergoing clinical trials for diagnosis and therapy. These reagents dominate the cancer-targeting field, as highlighted by the recent approval of the first engineered therapeutic antibodies by the FDA. Important advances have been made in the design, selection, and production of recombinant antibodies. The natural immune repertoire and somatic cell affinity maturation has been superseded by large antibody display libraries and rapid molecular evolution strategies. These novel libraries and selection methods have enabled the rapid isolation of high-affinity cancer-targeting and antiviral antibodies. In alternative strategies for cancer diagnosis and therapy, recombinant antibody fragments have been fused to radioisotopes, drugs, toxins, enzymes, and biosensor surfaces. Antibody-directed cancer pre-targeting followed by prodrug activation has proved a most promising therapeutic strategy. Multi-specific antibodies have been effective for cytotoxic T-cell recruitment and antibody fusion proteins have delivered enhanced immunotherapeutic and vaccination strategies. One of the major advantages of using antibodies is that it enables combination of diagnostics and therapeutics.

6.3.10 Combined Immunological and Nucleic Acid Tests

6.3.10.1 Combination of MAbs and RT-PCR

AdnaGen's proprietary technology enables detection of rare, CTCs—an early event in cancer metastasis. AdnaGen's two-step "Combinations Principle" involves (1)

cell isolation, where tumor cells are enriched by a MAb-mix linked to magnetic particles and mRNA is isolated from the selected tumor cells, and (2) molecular biological detection and analysis, whereby the isolated mRNA is transcribed into cDNA and a multiplex PCR is carried out for the analysis of tumor-associated gene expression. Due to the combination of different selection and tumor markers, both the heterogeneity of the tumor cells and possible individual or therapy-induced deviations in the expression patterns are taken into account. The two steps combine the benefits of immunoassay and nucleic acid testing and increase both the sensitivity and the specificity of cancer cell detection in body fluids such as blood and urine.

6.3.10.2 Immunobead RT-PCR

Immunomagnetic enrichment followed by RT-PCR (immunobead RT-PCR) is an efficient methodology to identify disseminated carcinoma cells in the blood and bone marrow. The RT-PCR assays must be both specific for the tumor cells and sufficiently sensitive to enable detection of single tumor cells. We need RT-PCR assays that are sensitive enough to detect very low numbers of captured carcinoma cells. As hematopoietic cells may be nonspecifically retained during immunomagnetic enrichment of epithelial cells, these RT-PCR markers also need to be tested for their specificity.

6.3.11 Assays for Determining Susceptibility to Cancer

There are few useful assays for predicting the risk of developing cancer. An assay of DNA repair defect could determine the susceptibility of an individual to cancer. Mutagen sensitivity was found to be an independent risk factor for other cancers. An assay such as the mutagen sensitivity assay may be a vital first step in measuring the overall DNA repair capacity of an individual.

6.3.12 Gene Expression Profiling in Cancer

Most of the cancer research during past few decades has been devoted to the analysis of genes that are expressed differently in tumor cells as compared with their normal counterparts. Intravital microscopy combined with green fluorescent protein (GFP) has provided powerful insight into gene expression in tumors. However, the optical techniques used are plagued by poor axial resolution. Multiphoton laser-scanning microscope can provide high 3D resolution of gene expression and function in deeper regions of tumors. Recently developed functional genomic approaches, such as DNA microarrays and SAGE, have enabled researchers to determine the expression

Table 6.4 Methods for comparison of gene expression profiling in tumor specimens

Method	Comments
mRNA differential display	Complicated and labor intensive
Serial analysis of gene expression (SAGE)	Limited for identifying unknown genes and requires genetic information
cDNA microarray	Very efficient but requires genetic information and expensive equipment
Large-scale cDNA sequencing	
Expressed sequence tag (EST) database comparison	
2D gel electrophoresis of cellular proteins	
Subtractive library construction	
Representational difference analysis (RDA)	
Suppression subtractive hybridization (SSH)	An efficient and versatile PCR-based method of identifying rare, tumor-specific transcripts

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level of every gene in a given cell population, which represents that cell population's entire transcriptome. Large-scale gene expression allows simultaneous study of thousands of genes of interest in a specific tissue/tumor of interest and the ability to identify expression signatures associated with functional phenotypes. Current methods for comparing gene expression profile changes in different tumor specimens are shown in Table 6.4. Some of these are described earlier in this report and others in the following text.

6.3.12.1 Microarrays for Gene Expression Profiling in Cancer

Microarray analysis can be used to search for global signatures of cancer metastases. Certain gene expression profiles correlate with primary versus metastatic cancers, regardless of tumor origin. Some primary tumors already contained a metastatic gene expression signature before they spread to other parts of the body, and this profile can be used to predict patient outcome. Using a cDNA array that contains 10,000–20,000 genes, clinical samples have been analyzed for gene expression differences between benign, local, and metastatic prostatic tissue. This approach identifies dozens of genes associated with prostate cancer.

Gene expression profiling using Affymetrix U133A GeneChips has been performed with RNA from mature aggressive B-cell lymphomas, including a core group of Burkitt's lymphomas to generate a molecular signature for Burkitt's lymphoma, and chromosomal abnormalities were detected with interphase FISH and array-based CGH (Hummel et al. 2006). The results produced a molecular definition of Burkitt's lymphoma, which clarified and extended the spectrum of the WHO criteria for Burkitt's lymphoma. In mature aggressive B-cell lymphomas without a gene signature for Burkitt's lymphoma, chromosomal breakpoints at the myc locus were associated with an adverse clinical outcome.

6.3.12.2 Serial Analysis of Gene Expression

SAGE technology involves taking a snapshot of mRNA population in a sample of interest in the form of small tags that correspond to fragments of those transcripts. Three principles underlie the SAGE technology:

1. One short oligonucleotide sequence from a defined location within a transcript ("tag") allows accurate quantitation.
2. Tag size (10–14 bp) is optimal for high throughput while maintaining accurate gene identification and quantitation.
3. The combined power of serial and parallel processing increases data throughput by orders of magnitude when compared to conventional expressed sequence analysis.

Important uses of this test include the study of differences in gene expression between cancer cells and their normal counterparts and identification of genes that may serve as useful diagnostic and prognostic markers. Differences in gene expression seen in SAGE translate directly into RNA differences as assessed by Northern blot analysis, and alterations identified in a few samples are consistent with data from a larger sample of primary tumor isolates. Gene expression monitoring by SAGE reduces the set of genes that are candidates for functional studies from tens of thousands that are expressed in cancers to a few hundred or less that show significant disparity under comparative conditions. Analysis of gene expression differences in treatment responders versus nonresponders could delineate differences between various patient populations and provide insight into the mechanism of action of different treatments. Gene expression patterns can also be useful in identifying new targets for therapeutic agents. SAGE helps to identify molecular differences, which correlate with adverse or beneficial response to drugs. Public sources of SAGE data, in particular through the Cancer Genome Anatomy Project, increase the value of this technology by making a large source of information on many tumors and normal tissues available for comparison. Variants of the original SAGE are LongSAGE and SuperSAGE, which have improved the technique with the capture of longer tags, enabling more confident identification of a source gene.

6.3.12.3 DNA Tags for Finding Genes Expressed in Cancer

A technique that tracks only switched-on genes in cells would enable the distinction between diseased and normal tissues and could point the way to new treatments. The tags are taken from mRNA transcripts—templates for protein production that are made when a gene is in action. Some studies have identified genes that are selectively active in cancer cells. Transcript analyses reveal differences between apparently identical tumors. This technique is particularly good at extracting information from the center of mRNAs, where a gene's function often resides.

Table 6.5 Important cancer tests based on gene signatures

Company	Test	Description	Status
Agendia	MammaPrint	Expression of 70 genes using Agilent arrays to determine risk of breast cancer recurrence	Cleared by FDA
Agendia	ColoPrint	Expression of 18 genes using Agilent arrays to determine a patient's risk of colon cancer recurrence	Approved
DiaGenic ASA	BCtect	Use of RT-PCR to study expression of 96 genes for the early detection of breast cancer	CE marked in EU, marketed in India
Foundation Medicine	FoundationOne™	NGS to interrogate hundreds of cancer-related genes from routine FFPE tumor samples	Launched in 2012
Genomic Health	Oncotype Dx Breast Cancer	Examines 21 genes using RT-PCR to ascertain recurrence risk and chemotherapy benefit for patients with ER-positive breast cancer	Launched under CLIA, run at company's lab
Genomic Health	Oncotype DX Colon Cancer	Examines a set of 16 genes using RT-PCR to ascertain recurrence risk for stage II colon cancer	Launched in 2010
Pathwork Diagnostics	Tissue of Origin test	Use of microarrays and PCR to study expression of a set of genes to determine a cancer's origin	Cleared for frozen tissue and FFPE

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6.3.12.4 Suppression Subtractive Hybridization

Suppression subtractive hybridization (SSH) is an efficient and versatile PCR-based method of identifying rare, tumor-specific transcripts. It has been used for the analysis of tumor specimens in several types of cancers including hepatocellular carcinoma (HCC), breast cancer, and glioblastoma multiforme (GBM). In applying this novel approach to breast cancer, a large panel of cDNA fragments encoding for the well-known tumor-associated surface antigens were identified, e.g., erb-B2, erbB3, and the urokinase receptor and, more importantly, for several clones overexpressed in breast cancer, whose cDNA fragments match the sequences of hypothetical transmembrane proteins with unknown function. The latter may represent novel tumor-specific targets (Di Cristina et al. 2007).

6.3.12.5 Cancer Tests Based on Gene Expression Profiling

Table 6.5 shows important cancer tests based on gene signatures.

6.3.13 Measurement of Telomerase Activity

Telomerase is abnormally reactivated in all major cancer types but is absent in most normal tissues. Telomerase activity can be analyzed using the telomeric repeat amplification protocol–hybridization protection assay, reverse transcription polymerase chain reaction (PCR), and Southern blot analysis.

Current methods for detecting telomerase activity follow a PCR-based telomeric repeat amplification protocol (TRAP) assay. TRAPeze/ELISA kits are marketed for quantitative detection of telomerase in tissues. A revised TRAP method incorporates a simplified TRAP–PCR assay using fluorochrome-labeled primer and the detection, visualization, and semiquantification of PCR products/telomerase activity with an automated laser-induced fluorescence capillary electrophoresis system. This method is highly reproducible for assessment and semiquantification of telomerase activity in a large number of hematological specimens.

Another way to detect telomerase activation is to look for elevated levels of RNA and/or protein components of telomerase in tumor cells. If the association between telomerase protein and cancer can be confirmed, it would be an improvement over current methods of histological diagnosis of cancer in some problematic situations. Telomerase can be assayed in cells that are extracted by less invasive methods than surgery, e.g., colonic washings for CRC. The TRAP assay is versatile and highly sensitive. It can detect a single cancer cell mixed with thousands of normal human cells and can be used to reveal the presence of cancer cells in almost any clinical specimen including biopsies, frozen sections, fine needle aspirates, brushes, washes, and biological fluids (e.g., urine, pancreatic juice, or blood). The original assay has been modified to reduce cost and facilitate the analysis of large numbers of samples. Telomerase estimation also has the potential for detection of precancerous lesions that may evolve into cancer. Apart from providing prognostic information, telomerase may also form the basis of anticancer drugs that could block telomerase activity.

A telomeric repeat elongation (TRE) assay directly measures telomerase activity as the telomeric elongation rate by biosensor technology using surface plasmon resonance. 5′-Biotinylated oligomers containing telomeric repeats are immobilized on streptavidin-pretreated dextran sensor surfaces in situ using the Biacore apparatus. The rate of TRE is calculated by measuring the SPR signals. This assay enables precise quantitative comparison of a wide range of human cells from somatic cells to carcinoma cells. TRE assay is suitable for practical use in the assessment of telomerase activity in preclinical and clinical trials of telomerase-based therapies, because of its reproducibility, rapidity, and simplicity.

However, TRAP–PCR or antibody-based radioimmunoassay is low throughput and not robust enough to easily accommodate the required statistical analysis to determine whether telomerase activity is a practical biomarker. A robot-assisted TRAP assay (RApidTRAP) of telomerase was developed for detection of this potential biomarker for early cancer detection. Measurements of human telomerase reverse transcriptase catalytic subunit (hTERT) mRNA were performed in concert

with measurement of telomerase activity. Comparison of high-throughput telomerase activity measurements using the robot and those performed manually was consistent in sensitivity and reproducibility. Using this combination of telomerase activity and hTERT mRNA measurements, the automated system showed improved efficiency over traditional TRAP–PCR methods.

6.3.14 Detection of Circulating Tumor Cells in Blood

Previously tumor antigens released from solid tumor cells into the bloodstream were used as biomarkers for longitudinal surveys in cancer patients. No routine, noninvasive diagnostic method was available to confirm the presence of cancer prior to clinical symptoms or manifestation at imaging studies. Blood samples are now analyzed for CTCs by nucleic acid methods to isolate tumor-associated or tumor-specific mRNA.

Not only are plasma DNA levels higher in cancer patients than in normal subjects, but they also correlate inversely with outcome and tend to fall with effective treatment. In many cancer patients, mutant DNA is the predominant subtype. Studies using PCR have identified ras mutations in the plasma of patients with pancreatic cancer, CRC, and hematological malignancies. Plasma DNA represents an attractive medium for diagnostic use and could potentially be used to identify patients at high risk of tumor recurrence in the absence of clinically detectable metastases. As a source of malignant DNA, plasma holds the potential of signaling the presence of residual metastatic disease; in contrast, other body fluids contain DNA only from the primary tumor. RT-PCR has been shown to be useful in detecting CTCs in adenocarcinomas such as prostate cancer but is not reliable in the case of epithelial tumors such as non-small-cell lung cancer (NSCLC). Detection of extremely low concentrations of rare CTCs in the blood is still a challenge. Automated digital microscopy is too slow to scan the large substrate areas. Some refinements for detection of cancer cells in blood are described in the following sections.

6.3.14.1 BEAMing Technology for Quantification of Circulating Tumor DNA

Inostics' BEAMing (Beads, Emulsions, Amplification, and Magnetics)—a digital technology that combines emulsion-based digital PCR with magnetic beads and flow cytometry—enables quantification of mutant DNA. It is a noninvasive, highly sensitive, real-time "liquid biopsy" for screening, prognosis, prediction, and monitoring of cancer. Key features are:

- Starting material: plasma, serum, or tissue (FFPE/frozen)
- Starting DNA amount: ≥1 genome equivalent (6.6 pg human genomic DNA)

- Detection capability: 0.01 % (mutant/total DNA) 1–4 (i.e., ≥1 mutant DNA molecule in 10,000 wild-type DNA molecules)
- Turnaround time of 2–4 days
- Available for analysis of 130 different mutations in >15 different cancer genes

6.3.14.2 Biochips/Microfluidics for Detection of CTCs

A microfluidic CTC-Chip device can capture CTCs that express epithelial cell adhesion molecules by using antibody-coated microposts. A high-throughput microfluidic mixing device, the herringbone-chip (HB-Chip), provides an enhanced platform for CTC isolation (Stott et al. 2010). The HB-Chip design applies passive mixing of blood cells through the generation of microvortices to significantly increase the number of interactions between target CTCs and the antibody-coated chip surface. Efficient cell capture was validated using defined numbers of cancer cells spiked into control blood, and clinical utility was demonstrated in specimens from patients with prostate cancer. CTCs were detected in 93 % patients with metastatic disease, and the tumor-specific TMPRSS2–ERG translocation was readily identified following RNA isolation and RT-PCR analysis. The use of transparent materials enables imaging of the captured CTCs using standard clinical histopathological stains, in addition to immunofluorescence-conjugated antibodies. Moreover, the 3D structure of the HB-Chip greatly facilitates scaled-up device production and will thus enable the initiation of larger-scale clinical studies.

CellSearch (Veridex) is currently the only CTC device approved by the FDA for tracking cancer progression. It captures CTCs on the basis of their affinity to antibodies EpCAM, which is found on tumor cells but not on blood cells. Using CellSearch, researchers have shown that the number of CTCs is a good predictor of survival and disease progression in people with metastatic breast, colon, and prostate cancers. However, a problem with this device is that the cell yields are very low, and they are not consistent. Moreover, the CellSearch system does not help in the selection of treatment for cancer.

6.3.14.3 CellTracks® AutoPrep® System

The CellTracks® AutoPrep® System (Janssen Diagnostics) is an automated sample preparation system for immunomagnetic cell capture and fluorescence staining of rare cells. It is used with the company's reagent kits to automate and standardize the isolation of CTCs. The CellTracks® Analyzer II is a semiautomated fluorescence microscope that is used to count and characterize the immunomagnetically selected cells based on the fluorescence signals of the cells. This technology for the quantification and characterization of rare cells is being used in drug development trials as efficacy biomarkers, for risk stratification and to monitor expression levels of proteins associated with targeted therapy. Detection of CTCs using immunomagnetics before initiation of first-line therapy in patients with metastatic breast cancer is

highly predictive of progression-free survival (PFS) and overall survival. Increased numbers of circulating endothelial cells (CECs) are observed in peripheral blood of cancer patients, which may contribute to tumor growth through the process of angiogenesis. Characterization of these cells by study of gene expression profiles of immunomagnetically enriched CECs may provide biomarkers to evaluate treatment efficacy. This technology can aid in appropriate patient stratification and design of personalized treatments.

6.3.14.4 CTCscope System for Detection of CTCs

CTCscope system (Advanced Cell Diagnostics) is based on the company's RNAscope technology for in situ RNA detection. The system identifies the molecular phenotypes of CTCs to guide treatment decisions and allows for the real-time monitoring of therapy response. Healthy blood samples spiked with tumor cell lines were used as a model system for the development and initial characterization of CTCscope. To demonstrate the feasibility of CTC detection in patient blood, duplicate blood samples were drawn from metastatic breast cancer patients for analysis by CTCscope as well as the CellSearch system to assess association of CTCs with the tumor biomarker CA 15-3 and PFS (Payne et al. 2012). Results showed that CTCscope detected CTC transcripts of eight epithelial markers and three epithelial–mesenchymal transition markers for increased sensitivity. CTCscope was used to detect CTCs with minimal enrichment and did not detect apoptotic or dead cells. In patient blood samples, CTCs detected by CellSearch, but not by CTCscope, were positively correlated with CA 15-3 levels. CTCs detected by either CTCscope or CellSearch predicted PFS. CTCscope offers unique advantages over existing CTC detection approaches. By enumerating and characterizing only viable CTCs, CTCscope provides additional prognostic and predictive information for monitoring of therapy.

6.3.14.5 ClearCell® FX System

ClearCell® FX System is driven by the CTChip® FR (Clearbridge BioMedics), which is a label-free method capable of isolating intact, viable CTCs from whole, unprocessed blood and counting them. The microfluidic biochip uses the unique differences in size and deformability of cancer cells compared to blood cells (Tan et al. 2010). Using physical structures placed in the path of blood specimens in a microchannel, CTCs which are generally larger and stiffer are retained, while most blood constituents are removed. The operations for processing blood are straightforward and permit multiplexing of the microdevices to concurrently work with different samples. The microfluidic device is optically transparent, enabling integration with existing laboratory microscopes, and immunofluorescence staining can be done in situ to distinguish cancer cells from hematopoietic cells. This also minimizes the use of expensive staining reagents, given the small size of the microdevice.

6.3.14.6 Fiber-Optic Array Scanning Technology

Fiber-optic array scanning technology (FAST) applies laser-printing techniques to the rare-cell detection problem. With FAST cytometry, laser-printing optics is used to excite 300,000 cells/s, and emission is collected in an extremely wide field of view, enabling a 500-fold speedup over ADM with comparable sensitivity and superior specificity. The combination of FAST enrichment and ADM imaging has the performance required for reliable detection of early-stage cancer in blood.

In a study of metastatic CRC patients, immunofluorescent staining with FAST was used to identify CTCs, with subsequent Wright–Giemsa and Papanicolaou staining (Marrinucci et al. 2010). The CTCs were compared to the corresponding primary and metastatic tumors. CRC CTCs showed marked intrapatient pleomorphism. In comparison to the corresponding tissue biopsies, cells from all sites showed similar pleomorphism, demonstrating that CRC CTCs retain the pleomorphism present in regions of solid growth. They also often retain particular cytomorphologic features present in the patient's primary and/or metastatic tumor tissue. This study provides an initial analysis of the cytomorphologic features of circulating CRC cells, providing a foundation for further investigation into the significance and metastatic potential of CTCs.

6.3.14.7 IsoFlux System

IsoFlux System (Fluxion Biosciences) utilizes a unique microfluidic design to provide automated cell introduction, trapping, sealing, whole-cell formation, and recording protocols. Stanford Cancer Institute is using IsoFlux System to isolate, recover, and analyze rare CTCs to provide a real-time "liquid biopsy" of samples for molecular analysis. The collaboration will focus on breast and lung cancer with a goal of subtyping different forms of the disease and developing treatments personalized to each individual patient.

6.3.14.8 Lab-on-Chip for the Isolation and Detection of CTCs

In 2010, the European Seventh Framework Project MIRACLE (Magnetic Isolation and moleculaR Analysis of single CircuLating and disseminated tumor cElls on chip) started to develop an operational lab-on-chip for the isolation and detection of CTCs and disseminated tumor cells (DTCs) in blood (www.miracle-fp7.eu). This lab-on-chip is an essential step toward faster and cost-efficient diagnosis of cancer. Whereas full tumor cell detection analysis can take more than a day, a lab-on-chip, integrating the many processing steps, would enable faster, easy-to-use, cost-effective detection of CTCs. They are minimally invasive, increasing the patient's comfort and the efficiency of healthcare.

In a preceding joint project by some of the partners (MASCOT FP6-027652), individual microfluidic modules for cell isolation, cell counting, DNA amplification,

and detection have been developed. Based on this expertise and strengthened by additional partners, the development of a fully automated lab-on-chip platform to isolate, count, and genotype CTCs is envisaged within the framework of the MIRACLE project. For genotyping, genetic material (i.e., the mRNA) will be extracted from the cells, and multiple cancer-related biomarkers will be amplified based on multiplex ligation-dependent probe amplification (MLPA) followed by their detection using an array of electrochemical sensors. Full integration of all steps requires innovative research and processing steps that need a combination of the multidisciplinary and unique expertise of the different project partners (ranging from microfluidics to interfacing, miniaturization, and integration skills). The resulting lab-on-chip tumor detection system will be well ahead of the current state of the art, revolutionizing cancer diagnostics and personalized cancer management.

6.3.14.9 MagSweeper

An immunomagnetic cell separator, the MagSweeper gently enriches target cells and eliminates cells that are not bound to magnetic particles (Talasaz et al. 2009). The isolated cells are easily accessible and can be extracted individually based on their physical characteristics to deplete any cells nonspecifically bound to beads. This device can process 9 mL of blood per hour and captures >50 % of circulating epithelial cells (CEpCs) from women with metastatic breast cancer. In contrast, no circulating epithelial cells were found in samples from healthy donors. The isolated CEpCs are all stored individually for further molecular analysis, which may provide candidate surrogate endpoints to diagnose, treat, and monitor malignancy directly from the blood samples.

6.3.14.10 Nano-Velcro to Capture CTCs for Diagnosis of Cancer

Although capturing CTCs aids in the diagnosis of cancer, most methods damage the cells in the process. Because CTCs number only in the hundreds or less, they are difficult to isolate from the billions of other cells in the blood. Assays that can capture CTCs have been approved for predicting survival in patients with metastatic breast cancer, prostate cancer, and CRC. An affordable nanoscale assay has been developed to quantify CTCs to improve cancer diagnosis and help understand how the disease spreads (Hou et al. 2013). This is a further development of the nanoscale Velcro-like material that can capture CTCs. However, simply capturing a cancer cell is not enough; it also needs to be analyzed against a panel of cancer biomarkers. The new method can release these cells, leaving them intact for further analysis such as genome sequencing. The technology is also cheaper, costing <$50 to manufacture, whereas comparable assays cost ~$1,000 per run. The device consisted of an array of silicon nanowires that are coated with antibodies, which bind to a protein that lines the outer membranes of some cancer cell types called EpCAM (Wang et al. 2009). This first-generation assay captured the targeted cells but released only about half of

them, and of those, only ~10 % of were viable, leaving the rest damaged. To improve on this, cell release was boosted by adding a temperature-sensitive polymer to the silicon nanowires. At 37 °C, the anti-EpCAM polymers grab tumor cells, and at 4 °C they release them. As a result, ~90 % of the released cells are undamaged. Similar to the first-generation assay, the new device only separates them with 40–70 % efficiency, but there is potential to boost efficiency further by adding a microfluidic component described previously (Wang et al. 2011). That component was a channel that increased blood flow across the nanostructured surface to improve separation efficiency. The assay is being validated using patient samples and a clinical trial is planned. Purity of the cell population is being improved by isolating and running samples through two assays in succession, which takes ~1 h.

6.3.14.11 Future Prospects of Detection of Cancer Cells in Blood

Rare CTCs present in the bloodstream of patients with cancer provide a potentially accessible source for detection, characterization, and monitoring of nonhematological cancers. Refinements of techniques for isolation of tumor cells in the blood and their characterization may emerge as a powerful diagnostic tool, facilitating oncogene analysis, early detection of cancer, localization of tumor, therapy selection, and determination of chemoresistance. Most promising of these technologies is the CTChip™, a microfluidic biochip for collection of CTC. Biochips for detection of CTCs may be suitable for point-of-care (POC) applications.

6.3.15 Epithelial Aggregate Separation and Isolation

In epithelial aggregate separation and isolation (EASI), smears of normal or tumor tissues are made, rapidly fixed, stained on glass slides, and identified by microscopic examination. Laser capture microdissection (LCM) is then performed, and nucleic acids are obtained for RNA and DNA assays. EASI is based on two principles: the inherent property of desmosome-enriched epithelial cells to separate from surrounding stromal tissue as tightly adherent clusters and the rapid alcohol-based fixation of thin layers of cells resulting in improved preservation of DNA, RNA, and proteins. This technique is applicable only to fresh tissues that have not been frozen or formalin-fixed. The advantages of this test are simplicity, speed, and low cost.

6.3.16 Proteomic Technologies for the Molecular Diagnosis of Cancer

The role of proteomic technologies in molecular diagnosis is introduced in Chap. 4. Limitations of the nucleic acid-based diagnostics were pointed out. Proteomic technologies have been applied with advantage in the diagnosis of cancer. The new

proteomic-based approaches to molecular diagnostics are aimed at the identification and investigation of protein markers in the actual histologically defined cell populations that are immersed in heterogeneous diseased tissue. It is envisioned that these investigations will eventually lead to novel diagnostic, prognostic, or therapeutic markers that can be applied to monitor therapeutic toxicity or efficacy. This is particularly important in cancer research where posttranslational modifications of a protein can specifically lead to the disease.

6.3.16.1 Proteomic Technologies for Tumor Biomarkers

2D PAGE followed by protein identification using mass spectrometry (MS) has been the primary technique for biomarker discovery in conventional proteomic analyses. This technique is uniquely suited for direct comparisons of protein expression and has been used to identify proteins that are differentially expressed between normal and tumor tissues in various cancers, such as liver, bladder, lung, esophageal, prostate, and breast. The disadvantages of the use of 2D PAGE for this purpose are as follows: (1) it requires a large amount of protein as starting material, making it unreliable for detecting and identifying low-abundance proteins, and (2) early-stage cancers are often small and contamination from surrounding stromal tissue that is present in the specimen can confound the detection of tumor-specific markers.

The introduction of LCM has greatly improved the specificity of 2D PAGE for biomarker discovery, as it provides a means of rapidly procuring pure cell populations from the surrounding heterogeneous tissue and also markedly enriches the proteomes of interest.

Surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS is an important tool for the rapid identification of cancer-specific biomarkers and proteomic patterns in the proteomes of both tissues and body fluids. It is useful in high-throughput proteomic fingerprinting of cell lysates and body fluids that uses on-chip protein fractionation coupled to TOF separation. Within minutes, sub-proteomes of a complex milieu such as serum can be visualized as a proteomic fingerprint or “barcode.” SELDI technology has significant advantages over other proteomic technologies in that the amounts of input material required for analysis are miniscule compared with more traditional 2D PAGE approaches. A number of studies have used SELDI technology to identify single disease-related biomarkers for several types of cancer. For example, a modified quantitative SELDI approach has been used to show that the levels of serum prostate-specific membrane antigen are significantly higher in patients with prostate cancer than in those with benign disease.

6.3.16.2 Affibodies as Contrast Agents for Imaging in Cancer

Affibody molecules are scaffold proteins, having a common frame of amino acids determining the overall fold or tertiary structure, but with each member characterized by a unique amino acid composition in an exposed binding surface determining binding specificity and affinity for a certain target. Affibody molecules represent a

new class of affinity proteins based on a 58-amino acid residue protein domain, derived from one of the IgG-binding domains of staphylococcal protein A. They combine small size (6.5 kDa) with high affinity and specificity. Picomolar binders have been obtained for Affibody molecules after selection of those with nanomolar affinities from an initial library and affinity maturation (Orlova et al. 2007). The small size and simple structure of Affibody molecules enable their production by chemical synthesis with homogeneous site-specific incorporation of moieties for further labeling using a wide range of labeling chemistries. The robustness and the refolding properties of Affibody molecules make them amenable to labeling conditions that denature most proteins, including incubation at pH 11 at 60 °C for up to 60 min. Affibody molecules meet the requirements for successful clinical use as imaging agents: (1) high-affinity binding to the chosen target; (2) short plasma half-life time; (3) rapid renal clearance for nonbound drug substance; and (4) high, continuously increasing tumor-to-organ ratios, resulting in high-contrast in vivo images shortly after injection of the diagnostic agent.

6.3.16.3 Aptamer-Based Technology for Protein Signatures of Cancer Cells

Aptamers are used to detect the protein signatures of cells. This is based on the tendency of short DNA molecules—oligonucleotides—to fold into shapes that bind to specific proteins. The aptamer hugs its protein in the same way as an antibody embraces a specific antigen. The technology can be used with biochips. Aptamer technology may provide a method for monitoring protein changes in the blood that can echo the onset of carcinogenesis, for example, in women with genetic risk of breast cancer associated with BRCA1 dysfunction.

A strategy using cell-based aptamer selection exploits the differences at the molecular level between any two types of cells for the identification of molecular signatures on the surface of targeted cells (Shangguan et al. 2006). A group of aptamers were generated for the specific recognition of leukemia cells. The selected aptamers can bind to target cells with an equilibrium dissociation constant (K_d) in the nanomolar-to-picomolar range. The cell-based selection process is simple, fast, straightforward, and reproducible and, most importantly, can be done without prior knowledge of target molecules. The selected aptamers can specifically recognize target leukemia cells mixed with normal human bone marrow aspirates and can also identify cancer cells closely related to the target cell line in real clinical specimens. The cell-based aptamer selection holds a great promise in developing specific molecular probes for cancer diagnosis and cancer biomarker discovery.

Archemix Corp. has developed a chip-based biosensor for multiplex analysis of protein analytes. The biosensor utilizes immobilized DNA and RNA aptamers, selected against several different protein targets, to simultaneously detect and quantify levels of individual proteins in complex biological mixtures. Aptamer–protein interactions recapitulate binding interactions seen in solution. Furthermore, specific detection and quantitation of cancer-associated proteins are possible in human

serum and in cellular extracts. It is expected that this technology would facilitate diagnosis of cancer by enabling direct detection of the expression and modification of proteins closely correlated with disease.

6.3.16.4 Aptamer Probes for In Vivo Diagnosis of Cancer

Aptamers have emerged as promising molecular probes for in vivo cancer imaging, but the reported "always-on" aptamer probes remain problematic because of high background and limited contrast. To address this problem, an activatable aptamer probe (AAP) was designed to target membrane proteins of living cancer cells and achieved contrast-enhanced cancer visualization inside mice (Shi et al. 2011). The AAP displayed a quenched fluorescence in its free state and underwent a conformational alteration upon binding to target cancer cells and could be specifically activated by target cancer cells with a dramatic fluorescence enhancement, and activated fluorescence signals were obviously achieved in the implanted tumor sites in mice. Compared to always-on aptamer probes, the AAP could substantially minimize the background signal originating from nontarget tissues, thus resulting in significantly enhanced image contrast and shortened diagnosis time to 15 min. The design concept can be widely adapted to other cancer cell-specific aptamer probes for in vivo molecular imaging of cancer.

6.3.16.5 Aptamers for Combined Diagnosis and Therapeutics of Cancer

High-affinity aptamers have been developed as targeted therapeutics for the diagnosis, imaging, staging, and treatment of cancers including breast, bladder, and stomach cancers, as well as a generic application for the treatment of abnormal growths with extensive potential future development. This method offers, apart from an immediate application in the diagnosis, imaging, and treatment of breast and other epithelial cancers, a generic application for the treatment of neoplastic disorders and extensive potential for future development. Combinatorial libraries have been used for the selection of aptamers that bind to a well-characterized and well-established cancer marker selectively and with high affinity. As part of their design, the aptamers are conjugated to ligands, molecules bearing binding sites for metal ions, to impart the therapeutic and diagnostic properties. In particular, stable chelation of technetium, rhenium, and yttrium radioisotopes result in novel radiopharmaceutical agents for imaging and selective cell kill as part of cancer diagnosis, imaging, and therapy. The use of paramagnetic gadolinium produces a novel, targeted MRI contrast agent that can achieve high local concentrations around the tumor site, thus offering high-definition imaging at lower gadolinium concentrations. The use of europium or terbium confers fluorescent properties to the aptamer complex, for use in diagnostic assays. These molecules offer significant advantages over existing antibody- and peptide-based recognition procedures in that they possess higher binding affinities to the target, leading to longer retention times and the ability to deliver a higher

payload of the metal ion precisely to the target with a lower overall dose of the agent. The size of these molecules leads to reduced immunogenicity and increased tumor penetration, further enhancing their efficacy while minimizing potential side effects. Their binding properties, retention in the tumor region, and clearance properties make them relevant tumor imaging probes.

6.3.16.6 Automated Image Analysis of Nuclear Protein Distribution

The organization of nuclear proteins is linked to cell and tissue phenotypes. When cells stop proliferating and undergo apoptosis or differentiation, distribution of proteins inside the nucleus changes. The proteins arrange themselves in a different pattern depending on whether the cell is malignant, has not developed a specific function, or is perfectly healthy and mature. In the case of malignant cells, this telltale shift in protein distribution is believed to occur before the cells proliferate.

Imaging methods have been developed to quantify the distribution of fluorescently stained nuclear protein NuMA found in the nuclei of human mammary cells using 3D cell culture (Knowles et al. 2006). When functioning properly, NuMA helps regulate various aspects of the cells' health. But when it malfunctions, the protein has been linked to leukemia and breast cancer. The technique, which couples state-of-the-art fluorescent imaging with computer-driven image analysis, distinguished proliferating nonneoplastic from proliferating malignant cells. So far, the technique has been used only to explore how changes to one nuclear protein, NuMA, impact the malignancy of a cell. In the future, the investigators will map a range of proteins and determine if changes to their spatial distribution are also a hallmark of cancer. Ultimately, this technique may join the many tools physicians use to diagnose and characterize cancer, such as hematoxylin/eosin staining, genetic tests, and medical imaging. This would enable physicians to detect aggressive cancers in their earliest stages and to better characterize a patient's cancer in order to match him or her with the most effective therapy.

6.3.16.7 Laser Capture Microdissection in Oncology

The most important use of LCM is in cancer in combination with proteomic technologies (Jain 2002). LCM is being used in the Cancer Genome Anatomy Program for systematic identification and cataloging of known and novel genes expressed during tumor development. It can be applied to any disease process, which is accessible through tissue sampling, such as premalignant lesions. LCM has played an important role in basic investigations of oncoproteomics. CD34, a heavily glycosylated transmembrane protein of ~110 kDa, is found in several neoplasms and has immunohistologic reactivity with anti-CD34 antibodies. LCM of CD34 immunohistologically reactive epithelioid sarcoma and nonreactive epidermal cells illustrates that this reactivity for anti-CD34 antibodies in apparently unrelated tumors is specific to tumor cells, not to shared epitopes on unrelated proteins.

Analysis on a microdissected epithelioid sarcoma shows that the ~110-kDa band is present in the sample containing tumor cells, which excludes the possibility that adjacent nonneoplastic cells are responsible for the immunoblot result.

High-density protein arrays, antibody arrays, and small molecular arrays, coupled with LCM, could have a substantial impact on proteomic profiling of human malignancies. In combination with techniques like expression library construction, cDNA array hybridization, and differential display, LCM enables the establishment of "genetic fingerprints" of specific pathological lesions, especially malignant neoplasms. This approach could help in establishing individualized treatments tailored to the molecular profile of a tumor.

Applied Biosystems® ArcturusXT™ LCM System (Life Technologies) is a unique microdissection instrument that offers the power of two lasers by combining laser capture and laser cutting into one modular platform. Its approach combines minimally invasive tissue sampling with ultrasensitive cell analysis technology called microgenomics. Using tissue samples from high-risk women, the researchers have used LCM to select 100–500 of the most abnormal appearing cells from the thousands of cells in the fine needle aspirate.

6.3.16.8 Layered Expression Scanning

Layered expression scanning (LES) is used for the comprehensive molecular analysis of tumor samples. This technology uses a layered array of capture membranes coupled to antibodies or DNA sequences to perform multiplex protein or mRNA analysis. Cell or tissue samples are transferred through a series of individual capture layers, each linked to a separate antibody or DNA sequence. As the biomolecules traverse the membrane set, each targeted protein or mRNA is specifically captured by the layer containing its antibody or cDNA sequence. The two-dimensional relationship of the cell populations is maintained during the transfer process, thereby producing a molecular profile of each cell type present. Practical value of the technique is demonstrated by analysis of prostate-specific antigen (PSA) protein, gelatinase A protein, and POV1 (PB39) cDNA. LES has now been licensed to 20/20 GeneSystems. As this technology progresses, it will have multiple applications for high-throughput molecular profiling of normal tissues and tumor samples.

6.3.16.9 Membrane-Type Serine Protease-1

The cell surface protease membrane-type serine protease-1 (MT-SP1, matriptase) is often upregulated in epithelial cancers. A study has shown that MT-SP1 is active on cancer cells and that its activity may be targeted in vivo for tumor detection (Darragh et al. 2010). A proteolytic activity assay with several MT-SP1-positive human cancer cell lines showed that MT-SP1 antibodies that inhibit recombinant enzyme activity in vitro also bind and inhibit the full-length enzyme expressed on cells. In contrast, in the same assay, MT-SP1-negative cancer cell lines were

inactive. Fluorescence microscopy confirmed the cell surface localization of labeled antibodies bound to MT-SP1-positive cells. To evaluate in vivo targeting capability, fluorescently labeled antibodies were administered to mice bearing tumors that were positive or negative for MT-SP1. Antibodies localized to MT-SP1-positive tumors enabling visualization of MT-SP1 activity, whereas MT-SP1-negative tumors were not visualized. These findings define MT-SP1 activity as a useful biomarker for epithelial cancers using a noninvasive antibody-based method.

6.3.16.10 Survivin and Molecular Diagnosis of Cancer

Survivin is a 16.5-kDa intracellular protein that belongs to the inhibitor of apoptosis (IAP) gene family. IAP molecules are found in the genomes of all metazoans. Several studies have consistently shown that expression of survivin inhibits cell death induced by various apoptotic stimuli. One of the most significant features of survivin is its differential distribution in cancer compared with normal tissues. The percentage of survivin-positive patients within the same tumor series is variable, reflecting the genetic heterogeneity of individual tumors. Cancer patients expressing survivin exhibit shortened survival, association with unfavorable markers of disease progression, accelerated rates of recurrences, and increased resistance to therapy. A simple immunohistochemical detection method applied to specimens might provide a quick predictive/prognostic indicator. Various cancer diagnostic strategies based on survivin are emerging. Cancer patients might not recognize survivin as a self-protein as it is absent in normal tissues and mount an immune response to it, providing a diagnostic tool. Circulating antibodies to survivin have been demonstrated in patients with cancer.

Survivin can also be detected directly in biological fluids of cancer patients, as a result of the shedding of tumor cells from the primary site. Survivin has been detected in the urine of all patients tested with new or recurrent bladder cancer (100 % specificity), whereas normal volunteers, patients with nonneoplastic genitourinary disease, or genitourinary cancers other than bladder test negative for urine survivin.

6.3.17 Biochip/Microarrays for Cancer Diagnosis

Biochips enable massively parallel molecular analyses to be carried out in a miniaturized format with a very high throughput. Tissue microarrays—composed of hundreds of tissue sections from different tumors—arrayed on a single glass slide. Applications of the various biochip technologies in cancer diagnosis include the following:

- Testing for disease predisposition by using SNP microarrays.
- Determination of cell types or tissues as well as clinical endpoints associated with molecular targets by using tissue microarrays.
- One can predict that individual cancer risks can, in the future, be estimated accurately by a microarray profile of multiple SNPs in critical genes.

Diagnostics of cancer will be facilitated by biochip readout of activity levels of thousands of genes and proteins. Tissue microarrays facilitate rapid evaluation of large-scale outcome studies. Realization of this potential depends on the ability to rapidly and precisely quantify the protein expression within each tissue spot. Algorithms have been developed that enable rapid, automated, continuous, and quantitative analysis of tissue microarrays, including the separation of tumor from stromal elements and the subcellular localization of signals. Validation studies using estrogen receptor (ER) in breast carcinoma show that automated analysis matches or exceeds the results of conventional pathologist-based scoring. Automated analysis and subcellular localization of beta-catenin in colon cancer identify two novel and prognostically significant tumor subsets, which are not detected by traditional pathologist-based scoring. Development of automated analysis technology empowers tissue microarrays for use in discovery-type experiments (more typical of cDNA microarrays), with the added advantage of inclusion of long-term demographic and patient outcome information.

Biochip diagnostics coupled with informatics solutions will form the basis of individualized treatment decisions for cancer patients. QIAGEN Marseille has developed biochips that can identify fusion gene transcripts in order to define the aggressiveness of leukemia and identify patients with poor prognosis who require aggressive treatment as well as monitoring of the treatment. The principle of this biochip is reverse transcription using random primers. PCR is performed from the gene of interest, and the detection step is performed on a DNA microarray. The proprietary test enables detection of molecular markers crucial for patient stratification and the assessment of leukemia therapy efficacy as well as MRD monitoring.

6.3.17.1 Role of DNA Microarrays in Gene Expression Profiling

A DNA microarray consists of tens of thousands of oligonucleotides or cDNAs of known sequences that are aligned in rows on a substrate, such as glass. RNA is isolated from the test sample (such as a tumor), reverse-transcribed into cDNA, and labeled with a fluorescent dye. RNA is also isolated from a reference sample (such as normal tissue), reverse-transcribed, and labeled with a different fluorophore. The samples are hybridized to the arrays, and the resulting fluorescence values reveal the relative levels of each RNA transcript in the test sample compared with the reference sample. Mathematical algorithms are used to cluster differences in expression patterns between sample sets. The cDNA arrays can reveal similarities and differences that are not necessarily evident from traditional approaches, such as morphologic or immunohistochemical analysis. The usefulness of this technique has been shown by demonstration of two subtypes, which can be defined by differing patterns of gene expression within morphologically homogeneous large-B-cell lymphomas.

DNA microarray-based gene expression is now used to study the tendency of a tumor to metastasize. A team at The Institute for Genomic Research (Rockville, MD) used a 19,200-element human cDNA microarray to profile transcription in three paired cell line models of colorectal tumor metastasis. By correlating expression patterns across these cell lines, they identified 176 genes that appear to be

differentially expressed (greater than twofold) in all highly metastatic cell lines relative to their reference. These findings indicate that several genes, some previously uncharacterized, may be causatively involved in, or at least prognostic of, metastasis.

6.3.17.2 Biochip Detection of FHIT Gene

FHIT (fragile histidine triad) tumor suppressor gene contains the most common human chromosomal fragile site at 3p14.2. Several genetic abnormalities in FHIT gene have been observed in cancer cell lines, uncultured tumors, and preneoplastic lesions. LOH associated with reduced FHIT gene expression has been observed in a high percentage of human tumors including breast, pancreas, esophagus, leukemia, and lung cancers. Detection of FHIT gene is important in cancer diagnostics. A unique multifunctional biochip for simultaneous detection of FHIT DNA and Fhit protein has been developed based on biomolecular recognition process using DNA and protein bioreceptors, Cy5-labeled probes, and laser excitation. This biochip is useful for various applications in biological and clinical laboratories. The advantages of this technology are reduced time scale, requiring less initial sample and concentration of reagents, a greater resolution of detection and specificity, and low cost.

6.3.18 Multiplexed Single-Cell Analysis of FFPE Cancer Tissue Samples

A multiplexed fluorescence microscopy method (MxIF) has been described for quantitative, single-cell, and subcellular characterization of multiple analytes in FFPE cancer tissue samples (Gerdes et al. 2013). Chemical inactivation allows reuse of fluorescent dyes after each staining and imaging cycle. MxIF is compatible with total and phosphoprotein detection as well as DNA FISH. Alignment of nuclear counterstain-derived points enables accurate computational analysis of sequential images. Individual cells, plasma membrane, cytoplasm, nucleus, tumor, and stromal regions are segmented to achieve cellular and subcellular quantification of multiplexed targets. Comparison of pathologists' scoring of diaminobenzidine staining of serial sections and automated MxIF scoring of a single section, human EGFR-2, ER, p53, and androgen receptor staining by diaminobenzidine yielded similar results. Single-cell staining patterns of protein antigens by MxIF in CRC subjects reveal extensive tumor heterogeneity, and cluster analysis of divergent signaling through ERK1/2, S6 kinase 1, and 4E-binding protein 1 provides insights into the spatial organization of target of rapamycin and MAPK signal transduction. Results suggest potential applications of MxIF in cancer biology research, anticancer drug discovery, and cancer diagnostics.

6.3.19 Nanobiotechnology for Early Detection of Cancer

Nanobiotechnology (see Chap. 9) offers a novel set of tools for detection of cancer. It will contribute to early detection of cancer as follows:

- It can complement existing technologies and make significant contributions to cancer detection, prevention, diagnosis, and treatment.
- It would be extremely useful in the area of biomarker research and provide additional sensitivity in assays with relatively small sample volumes.
- Examples of applications of nanobiotechnology in cancer diagnostics include quantum dots (QDs) and use of nanoparticles for tumor imaging.

6.3.19.1 Detection of Nanoparticle Self-Assembly in Tumors by MRI

A technique that allows nanoparticles to group together inside malignant tumors, creating masses with enough of a magnetic signal to be detectable by MRI, may help early detection of cancer (Harris et al. 2006). The procedure in animal experiments involves injecting iron oxide nanoparticles into the blood circulation from where they enter tumors. Once inside the tumor, the nanoparticles can be triggered to self-assemble by a mechanism involving certain enzymes or proteases. The resulting nanoparticle clumps are too large to get back into the circulation. Further, the clumps have a stronger magnetic signal than do individual nanoparticles, allowing detection by MRI. The technique initially is being used to study breast tumors. It will eventually be applied to many different types of cancers and to study the "triggers" that turn a benign mass in the body into a malignant tumor. Nanoparticles also hold the promise of carrying medicines that could kill cancer cells.

6.3.19.2 Differentiation Between Normal and Cancer Cells by Nanosensors

Rapid and effective differentiation between normal and cancer cells is an important challenge for the diagnosis and treatment of tumors. A nanoparticle array-based system has been described for identification of normal and cancer cells based on a "chemical nose/tongue" approach that exploits subtle changes in the physicochemical nature of different cell surfaces (Bajaj et al. 2009). Differential interactions with functionalized nanoparticles are transduced through displacement of a multivalent polymer fluorophore that is quenched when bound to the particle and fluorescent after release. This sensing method can rapidly (minutes/seconds) and effectively distinguish (1) different cell types; (2) normal, cancerous, and metastatic human breast cells; and (3) isogenic normal, cancerous, and metastatic murine epithelial cell lines.

6.3.19.3 Magnetic Nanoparticle Probes

An ultrasensitive method for detecting protein analytes relies on magnetic nanoparticle probes with antibodies that specifically bind a target of interest and nanoparticle probes that are encoded with DNA that is unique to the protein target of interest and antibodies that can sandwich the target captured by the microparticle probes. Magnetic separation of the complexed probes and target followed by dehybridization of the oligonucleotides on the nanoparticle probe surface allows the determination of the presence of the target protein by identifying the oligonucleotide sequence released from the nanoparticle probe. Because the nanoparticle probe carries with it a large number of oligonucleotides per protein-binding event, there is substantial amplification, and PSA can be detected at 30 aM concentration. Alternatively, a PCR on the oligonucleotide barcodes can boost the sensitivity to 3 aM. Comparable clinically accepted conventional assays for detecting the same target have sensitivity limits of 3 pM, six orders of magnitude less sensitive than what is observed with this method.

6.3.19.4 Quantum Dots for Early Detection of Cancer

There is considerable interest in the use of QDs as inorganic fluorophores, owing to the fact that they offer significant advantages over conventionally used fluorescent markers. For example, QDs have fairly broad excitation spectra—from ultraviolet to red—that can be tuned depending on their size and composition. At the same time, QDs have narrow emission spectra, making it possible to resolve the emissions of different nanoparticles simultaneously and with minimal overlap. Last, QDs are highly resistant to degradation, and their fluorescence is remarkably stable. QDs, coated with a polyacrylate cap and covalently linked to antibodies or to streptavidin, have been used for immunofluorescent labeling of breast cancer marker Her2. Labeling is highly specific and was brighter and more stable than that of other fluorescent markers.

A QD-based test has been developed for detection of DNA methylation, which contributes to carcinogenesis by silencing key tumor suppressor genes. The ultrasensitive and reliable nanotechnology assay, MS-qFRET (fluorescence resonance energy transfer), can detect as well as quantify DNA methylation (Bailey et al. 2009). Bisulfite-modified DNA is subjected to PCR amplification with primers that would differentiate between methylated and unmethylated DNA. QDs are then used to capture PCR amplicons and determine the methylation status via FRET. The specific target of the test is DNA methylation which occurs when methyl attaches to cytosine, a DNA building block. When this happens at specific gene locations, it can stop the release of tumor-suppressing proteins; cancer cells then more easily form and multiply. The method involves singling out the DNA strands with methyl attachments through bisulfite conversion, whereby all non-methyl segments are converted into another nucleotide. Copies of the remaining DNA strands are made, two molecules (a biotin protein and a fluorescent dye) are attached at

either end, and the strands are mixed with QDs that are coated with a biotin-attractive chemical. Up to 60 DNA strands are attracted to a single QD. An UV light or blue laser activates the QDs, which pass the energy to the fluorescent molecules on the DNA strands which then light up and are identifiable via a spectrophotometer, which both identifies and can count the DNA methylation.

Key features of MS-qFRET include its low intrinsic background noise, high resolution, and high sensitivity. This approach detects as little as 15 pg of methylated DNA in the presence of a 10,000-fold excess of unmethylated alleles, enables reduced use of PCR (as low as eight cycles), and allows for multiplexed analyses. The high sensitivity of MS-qFRET enables one-step detection of methylation at PYCARD, CDKN2B, and CDKN2A genes in patient sputum samples that contain low concentrations of methylated DNA, which normally would require a nested PCR approach.

The direct application of MS-qFRET on clinical samples offers great promise for its translational use in early cancer diagnosis, and prognostic assessment of tumor behavior, as well as monitoring response to therapeutic agents. Gene DNA methylation indicates a higher risk of developing cancer and is also seen as a warning sign of genetic mutations that lead to development of cancer. Moreover, since different cancer types possess different genetic markers, e.g., lung cancer biomarkers differ from leukemia, the test should identify which cancer a patient is at risk of developing. This test could be used for frequent screening for cancer and replacing traditionally invasive methods with a simple blood test. It could also help determine whether a cancer treatment is effective and thus enable personalized chemotherapy.

6.3.20 Molecular Imaging of Cancer

Molecular imaging of cancer is important not only as a guide to assessment of treatment effects but also as a guide to surgical excision. Several technologies have been used including fluorescence, optical systems, and imaging techniques such as MRI and PET.

6.3.20.1 In Vivo Tumor Illumination by Adenoviral GFP

Cancer surgery requires the complete and precise identification of malignant tissue margins including the smallest disseminated lesions. Internal GFP fluorescence can intensely illuminate even single cells but requires GFP sequence transcription within the cell. Introducing and selectively activating the GFP gene in malignant tissue in vivo is made possible by the development of OBP-401, a telomerase-dependent, replication-competent adenovirus expressing GFP. This potentially powerful adjunct to surgical navigation was demonstrated in mouse models that represent difficult surgical challenges—the resection of intraperitoneal disseminated human colon cancer (Kishimoto et al. 2009). Only the malignant tissue

fluoresced brightly, and fluorescence-guided surgery enabled resection of all tumor nodules labeled with GFP by OBP-401. The data in this report suggest that adenoviral GFP labeling tumors in patients can enable fluorescence-guided surgical navigation.

6.3.20.2 PET for In Vivo Molecular Diagnosis of Cancer

During the past decade, PET has been increasingly developed for imaging and quantifying molecular mechanisms in oncology. The technique uses radionuclides to label molecules, which can then be imaged in man. PET with ^{18}F-fluorodeoxyglucose (FDG) as a radioactive tracer for glucose metabolism is currently an effective and highly utilized tool for the diagnosis and management of cancer. Growing tumors consume glucose and show up as bright spots on PET, which can disappear a week after a patient begins chemotherapy, signaling possible remission. Waiting for demonstrable tumor shrinkage on computed tomography scans takes another 6 months. Seeing whether a patient actually lives longer could take years. Usually, changes in PET results are not dramatic enough to be seen by the naked eye, but the signal must be interpreted through a series of calculations gauging the concentration of a radioactive tracer.

Continuing technological improvements in imaging, including higher resolution, better attenuation correction, and multimodality image registration, will further improve the efficacy of this method. The most significant improvements will come from the wide variety of tracers now being developed to image other metabolic pathways and to identify cancer by specific biochemical, physiological, and genetic characteristics. Developments in PET mean that a wider range of molecules can now be labeled with isotopes, and old and new molecular targets for anticancer therapy can be probed, imaged, and quantified in vivo in man. The inherent sensitivity and specificity of PET are unrivalled because it can image molecular interactions and pathways, providing quantitative kinetic information down to the subpicomolar level. Molecular imaging has the potential to assist in the optimization of molecular-based targeted therapies in cancer and to investigate the function of the genome.

6.3.20.3 Radiolabeled Peptide-Based Targeting Probes for Cancer Imaging

Because of the low targeting efficiency of nonspecific contrast agents, there is a need for molecularly targeted imaging probes. Overexpression of peptide receptors in many human cancers has enabled the development of tumor-specific targeting molecules for imaging and therapy of cancers. The use of solid-phase peptide synthesis and the availability of a wide range of bifunctional chelating agents for the radiolabeling of bioactive peptides with radionuclides have produced a wide variety of useful radiopharmaceutical molecules (Ruzza and Calderan 2011).

6.3.20.4 Optical Systems for In Vivo Molecular Imaging of Cancer

A new class of molecular specific contrast agents for molecular imaging is based on gold nanoparticles attached to probe molecules with high affinity for specific cellular biomarkers. The application of gold bioconjugates for vital imaging of precancers was shown by using cancer cell suspensions, 3D cell cultures, and neoplastic fresh cervical biopsies. Gold conjugates could be delivered topically for imaging throughout the whole epithelium. These contrast agents have potential to extend the ability of vital reflectance microscopies for in vivo molecular imaging. They can potentially enable combined screening, detection, and therapy of disease using inexpensive imaging systems; such tools could allow mass screening of diseases such as cancer in resource-poor settings.

Celsense Inc. has formulated a novel a dual-mode contrast agent through the addition of an NIR probe to a perfluorocarbon (PFC)-based 19F MRI agent, which labels inflammatory cells in situ. A single PFC-NIR imaging agent enables both a qualitative, rapid optical monitoring of an inflammatory state and a quantitative, detailed and tissue-depth-independent imaging. The feasibility of in vivo optical imaging of the inflammatory response was demonstrated in a subcutaneous murine breast carcinoma model (Balducci et al. 2013). Ex vivo optical imaging was used to quantify the PFC-NIR signal in the tumor and organs, and results correlated well with quantitative 19F NMR analyses of intact tissues. 19F MRI was employed to construct a 3D image of the cellular microenvironment at the tumor site. Flow cytometry of isolated tumor cells was used to identify the cellular localization of the PFC-NIR probe within the tumor microenvironment. Contrast is achieved through the labeling of host cells involved in the immune response, but not tumor cells. The major cellular reservoir of the imaging agent were tumor-infiltrating CD11b+ F4/80low Gr-1low cells, a cell subset sharing immunophenotypic features with myeloid-derived suppressor cells, which are recruited to sites of inflammation and are implicated in immune evasion and tumor progression. This PFC-NIR contrast agent coupled to noninvasive, quantitative imaging techniques could serve as a valuable tool for evaluating novel anticancer agents. In order to use such optical systems to image molecular features of cancer, it will be necessary to deliver sufficient contrast agent to tissue so that a reasonable signal-to-noise ratio can be obtained. Before contrast agents can be used in human subjects, extensive animal studies must be carried out to evaluate any potential toxicity of these contrast agents and delivery formulations.

Fluorescent Affibody-based probes have been used for in vivo analysis of human epidermal growth factor receptor-2 (HER2) receptors using NIR optical imaging that do not interfere with binding of the therapeutic agents to these receptors (Ardeshirpour et al. 2013). Two types of breast carcinoma xenografts with significant differences in HER2 expression were analyzed in the mouse model. The use of a kinetic model to analyze temporal variations of the fluorescence intensity in the tumor area after two subsequent injections enabled quantitative assessment of the difference in HER2 expression levels for two tumor types, and results were substantiated by ELISA ex vivo assays of HER2 expression in the same tumors.

Imaging systems with potential to impact oncology practice are shown in Table 6.6.

Table 6.6 Impact of in vivo molecular imaging of cancer on oncology practice

Change current clinical practice
Screening
Early detection
Margin delineation
Follow tissue response to molecular therapies such as chemoprevention
Change clinical practice of the future
Identify lesions at risk of progression by phenotype and molecular profile
Enable rational choice of therapeutic agent
Allow rapid molecular assessment of efficacy of treatment
Development of personalized cancer therapy

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6.3.21 Detection of Micrometastases

Micrometastasis is the spread of cancer away from a primary tumor that is not detectable by routine screening tests. This is because the metastasis is too limited to have created enough mass to be observed using current examination techniques. Micrometastases are also considered to be a cause of recurrence after curative surgery for cancer. Micrometastases can also be detected in peripheral blood samples. Molecular biological detection of micrometastasis following curative surgery of gastric carcinoma can be performed on lavage cytology specimens using RT-PCR of carcinoembryonic antigen (CEA) mRNA or telomerase activity assay. Approximately 50 % of early-stage cervical cancers shed tumor cells to the pelvic lymph nodes as micrometastases. The amount of cytokeratin-19 expression as detected by RT-PCR can be related to clinicopathologic features. The effective treatment of early-stage recurrent cancer can prolong patient survival and reduce healthcare costs associated with late-stage cancer treatments.

6.3.22 Detection of Origin in Cancers of Unknown Primary

Almost one-third of advanced cancers presents with metastases at time of diagnosis. In the majority of cases, the organ site of primary lesion becomes evident shortly after clinical, pathological, and radiometabolic evaluations. A patient is considered to have cancer of unknown primary (CUP) origin when a tumor is detected at one or more metastatic sites and clinical evaluation, as well as available cancer diagnostics, fails to identify a primary tumor. Metastatic CUP constitutes 3–5 % of all cancers and poses a major challenge in diagnosis. More than 50 % of CUP patients present with multiple sites of involvement, while the rest have a single site, most commonly liver, bone, lung, or lymph nodes. The regression or dormancy of the primary tumor, the development of early, uncommon systemic metastases, and the resistance to therapy are hallmarks of CUP. There are approximately 200,000 CUP patients in the USA.

IHC is often the only standard method by which a putative primary origin can be postulated. In some cases, IHC algorithms enable the identification of a primary site with adequate accuracy, but the knowledge is inadequate to definitely distinguish two different groups, those with and without IHC-solved primary. If IHC is inconclusive, assays for molecular profiling are considered. There are currently several commercial tests available with gene expression-based assays that classify tumors of unknown or uncertain origin and claim prediction accuracies in known primary cancers between 80 and 90 % (Stella et al. 2012):

- Pathwork's microarray for mRNA
- bioTheranostics' RT-PCR for mRNA
- Rosetta Genomics' miRNA-based miRview mets2 assay
- Prometheus' RT-PCR for miRNA

6.4 Molecular Diagnosis of Cancers of Various Organs

The following sections describe potential applications of molecular techniques to cancer diagnosis, based on the organ involved and the type of cancer. Common cancers for which these diagnostic methods may be employed include brain tumors, breast carcinoma, cervical cancer, CRC, head and neck cancer, leukemia, lung cancer, melanoma, ovarian tumors, prostate cancer, and urinary bladder cancer.

6.4.1 Brain Tumors

Routine diagnosis of brain tumors is based on in vivo brain imaging and histological examination. In the past, most of the genetic studies of tumors involved cytogenetic analysis. These studies can detect gross chromosomal abnormalities, numerical or structural. Numerical abnormalities involve the loss or gain of parts of chromosomes or the whole chromosomes or the gain of an entire complement of chromosomes. Structural alterations include deletions, duplications, inversions, and translocations. Karyotype analysis requires that the cells are actively dividing and may not be easily applicable to solid tumors with a low mitotic index. Culturing cells can introduce cytogenetic aberrations. Limitations of cytogenetic techniques are that they can detect changes in the genome only if the size of the region involved is greater than 3,000–5,000 kb. Loss of genetic material detected by these techniques may affect many genes simultaneously. The findings may give a clue of where to look for more detailed changes by molecular methods.

6.4.1.1 Molecular Diagnostic Methods for Brain Tumors

Various methods used for molecular diagnosis of brain tumors include RFLP markers, polymorphic DNA probes to detect LOH, gene expression, FISH, RT-PCR,

and SAGE. Various genetic alterations seen are listed below and described in more detail elsewhere (Jain 2013c).

- Losses of alleles at loci on chromosomes 1, 9, 10, 13, 17, 19, and 22
- Losses of p53 tumor suppressor genes
- Deletion of p15, p16
- mdm2 amplification (found in 15 % of malignant gliomas)
- Increased sensitivity of the growth factor receptors to endogenous growth factors: fibroblast growth factor, platelet derived growth factor, tumor growth factor, and epidermal growth factor (EGF)
- MMAC1 (mutated in multiple advanced cancers 1), a gene involved in the progression of glioma to its most malignant form
- MAGE (melanoma-associated antigen gene)-E1, a glioma-specific member of MAGE family

6.4.1.2 Glioblastoma Multiforme

The most common primary malignant tumor of the brain in adults is GBM. Insulin-like growth factor-binding protein 2 (IGFBP2) has been shown to be overexpressed in GBM and contributes to the invasiveness of tumor as well as correlates with histological grade and survival of patients. The expression of two other IGFBP family members in diffuse gliomas, IGFBP3 and IGFBP5, has been studied using IHC applied to a tissue array constructed from tumor tissue (Wang et al. 2006). Expression of IGFBP5 correlated significantly with glioma histological grade; 83 % of GBMs (WHO grade IV) were positive for IGFBP5, which was significantly higher than WHO grade III gliomas (41 %) or WHO grade II gliomas (18 %). This study demonstrates that the expression of IGFBP5, but not IGFBP3, increases with progression of malignancy of the glioma. Codon 72 polymorphism in tumor suppressor gene p53 is associated with onset of GBM in young patients (El Hallani et al. 2009).

A comprehensive analysis using next-generation sequencing technologies has led to the discovery of a variety of genes that were not known to be altered in GBMs (Parsons et al. 2008). There were recurrent mutations in the active site of isocitrate dehydrogenase 1 (IDH1) in 12 % of GBM patients; these occurred in a large fraction of young patients and in most patients with secondary GBMs and were associated with an increase in overall survival. These studies demonstrate the value of unbiased genomic analyses in the characterization of human brain cancer and identify a potentially useful genetic alteration for the classification and targeted therapy of GBMs.

Mutations of NADP-dependent isocitrate dehydrogenases (IDH) encoded by IDH1 and IDH2 genes occur in a majority of several types of malignant gliomas (Yan et al. 2009). The study identified mutations that affected amino acid 132 of IDH1 in more than 70 % of WHO grade II and III astrocytomas and oligodendrogliomas and in glioblastomas that developed from these lower-grade lesions. Tumors without mutations in IDH1 often had mutations affecting the analogous amino acid (R172)

of the IDH2 gene. Tumors with IDH1 or IDH2 mutations had distinctive genetic and clinical characteristics, and patients with such tumors had a better outcome than those with wild-type IDH genes. Persons with certain tumors that carry these genetic alterations appear to survive at least twice as long as those without them. Further research on the genes could also lead to more precise diagnosis and treatments.

6.4.1.3 Circulating Microvesicles as Biomarkers of Glioblastoma

Glioblastoma tumor cells release microvesicles (exosomes) containing mRNA, miRNA, and angiogenic proteins, which are taken up by normal host cells, such as brain microvascular endothelial cells. By incorporating an mRNA for a reporter protein into these microvesicles, it was demonstrated that messages delivered by microvesicles are translated by recipient cells (Skog et al. 2008). These microvesicles are also enriched in angiogenic proteins and stimulate tubule formation by endothelial cells. Tumor-derived microvesicles therefore serve as a means of delivering genetic information and proteins to recipient cells in the tumor environment. Glioblastoma microvesicles also stimulated proliferation of a human glioma cell line, indicating a self-promoting aspect. mRNA mutant/variants and miRNAs characteristic of gliomas could be detected in serum microvesicles of glioblastoma patients. The tumor-specific EGFRvIII was detected in serum microvesicles from several glioblastoma patients and can be considered a biomarker. Thus, tumor-derived microvesicles represent a new way of getting information about a cancer without a biopsy, offering a means of choosing the best therapy, seeing how a patient responds to treatment, and possibly offering a way to deliver therapies back to the tumor. Exosome Diagnostics Inc. has licensed the technology for further development.

6.4.1.4 Combination of Neuroimaging and DNA Microarray Analysis in GBM

Combined neuroimaging and DNA microarray analysis have been used to create a multidimensional map of gene expression patterns in GBM that provides clinically relevant insights into tumor biology (Diehn et al. 2008). Tumor contrast enhancement and mass effect can predict activation of specific hypoxia and proliferation gene expression programs, respectively. Overexpression of epidermal growth factor receptor (EGFR), a receptor tyrosine kinase and potential therapeutic target, has also been directly inferred by neuroimaging and validated in an independent set of tumors by IHC. Furthermore, imaging provides insights into the intratumoral distribution of gene expression patterns within GBM. An "infiltrative" imaging phenotype can identify and predict patient outcome. Patients with this imaging phenotype have a greater tendency toward having multiple tumor foci and demonstrate significantly shorter survival than their counterparts. This study demonstrates a simple, widely applicable method for discovering imaging biomarkers that are associated

with underlying gene expression signatures. This approach facilitates the association of complex molecular signatures with readily identifiable imaging characteristics. These findings provide an in vivo portrait of genome-wide gene expression in GBM and offer a potential strategy for noninvasively selecting patients who may be candidates for individualized therapies.

6.4.1.5 Medulloblastoma

Medulloblastomas, embryonal tumors of the central nervous system, for example, are the most common malignant brain tumor of childhood, but their pathogenesis is unknown, and patients' response to therapy is difficult to predict. Scientists at the Massachusetts General Hospital (Boston, MA) have approached these problems by developing a classification system based on DNA microarray gene expression data derived from patient samples. They demonstrated that medulloblastomas are molecularly distinct from other brain tumors including malignant gliomas. They further showed that the clinical outcome of children with medulloblastomas is highly predictable on the basis of the gene expression profiles of their tumors at diagnosis.

6.4.1.6 Advantages and Limitations of Molecular Diagnosis of Brain Tumors

The potential advantages of developing molecular testing for brain tumors are:

- These methods will increase our understanding of the biology of brain tumors and enable an improved classification and new approaches to treatment. It may be possible to determine the cells of origin of some tumors.
- Targets for alternative therapies for brain tumors will include defective tumor suppressor genes. A gene therapy strategy may be used to correct or replace the defective gene.
- Diagnosis may be possible with a very small amount of biopsy tissue or an archival tissue specimen.
- Genotyping of brain tumors may have an application in stratifying patients for clinical trials of various novel therapies.
- Determination of prognosis by immunohistochemical evaluation of molecular biological parameters.

Some limitations of molecular approaches for diagnosis of brain tumors are:

- There is a subset of particularly aggressive high-grade gliomas with no currently known molecular genetic abnormalities.
- Very little information is available for malignant tumors except GBM.
- In some studies, the overexpression of p53 does not correlate with survival. The role that this gene plays in the development and progression of gliomas remains unclear.

6.4.2 *Breast Cancer*

Early detection of breast cancer is important for management and prognosis. Clinical breast examination, mammography, and ultrasound are the most commonly used currently for screening for breast cancer. However, these methods have limitations as they can yield false-negative results. Even after careful clinical and mammographic evaluation, cancer is found in the contralateral breast in up to 10 % of women who have received treatment for unilateral breast cancer. MRI can detect cancer in 3 % of the contralateral breast that is missed by mammography and clinical examination at the time of the initial breast cancer diagnosis (Lehman et al. 2007). MRI scans are recommended in women who have breast cancer or are at high risk for it.

6.4.2.1 Breast Cancer Genes

Approximately 5–10 % of cases of breast cancer are due to inheritance of a mutated copy of one of the two genes known as BRCA1 and BRCA2. BRCA1 mutations confer a risk of 56 % for breast cancer by the age of 70, regardless of the family history. While these risks are lower than earlier estimates, which pegged the risk of breast cancer at 85 % for women with such mutations, they are still quite high. In the general population, however, BRCA1 mutations are found in only 6 % of women diagnosed as having breast cancer before the age of 35 years. Mutations in certain genes that regulate the cell cycle, such as p16 and p53, are frequently found in human cancers, including breast cancer. Inactivation of BRCA2 and of p53 combines to mediate mammary tumorigenesis and indicates that disruption of the p53 pathway is pivotal in BRCA2-associated breast cancer. A third gene mutation, CHEK2, is linked to high rates of breast cancer, although it is not as important as the BRCA1 and BRCA2 mutations in indicating breast cancer risk (Weischer et al. 2007). However, women may benefit from screening for the mutation, which was found in 1 % of white, Northern European women. The study only included Danish women, leaving questions about its prevalence in black and Hispanic women unanswered. In the study, 0.5 % of Danish women had the mutation, and 12 % of them developed breast cancer, compared to 5 % of the women who did not carry the mutation. Women with the mutation who were over 60, overweight, and taking hormone replacement therapy had a 24 % chance of developing breast cancer in 10 years.

Another gene for breast and ovarian cancer has been identified, which explains the link between hereditary and “sporadic” (non-inherited) forms of these cancers. The gene has been named EMSY and maps to chromosome 11q13.5, a region known to be involved in breast and ovarian cancer. EMSY gene is amplified almost exclusively in sporadic breast cancer (13 %) and higher-grade ovarian cancer (17 %). In addition, EMSY amplification is associated with worse survival, particularly in node-negative breast cancer, suggesting that it may be of prognostic value. The remarkable clinical overlap between sporadic EMSY amplification and

familial BRCA2 deletion implicates a BRCA2 pathway in sporadic breast and ovarian cancer.

Known susceptibility genes account for less than 25 % of the familial risk of breast cancer, and the residual genetic variance is likely to be due to variants conferring more moderate risks. To identify further susceptibility alleles, a two-stage genome-wide association study was conducted in 4,398 breast cancer cases and 4,316 controls, followed by a third stage in which 30 SNPs were tested for confirmation in 21,860 cases and 22,578 controls from 22 studies (Easton et al. 2007). SNPs in five novel independent loci exhibited strong and consistent evidence of association with breast cancer. Changes in four of these genes (FGFR2, TNRC9, MAP3K1, and LSP1) raise the risk of breast cancer significantly. Several are found in many men and women. More than 60 % of the women in the USA probably carry at least one of the mutations in FGFR2. These discoveries concern the most important genes associated with breast cancer since BRCA1 and BRCA2 were identified.

Another study looked at more than 2,200 women of European ancestry and found four common mutations in FGFR2 associated with breast cancer in women after menopause who do not have known relatives with breast cancer (Hunter et al. 2007). The mutations raise the risk of breast cancer risk by 20 % if they carry one copy of the gene and by 60 % if they carry two copies. And close to 60 % of the women they studied carried at least one copy. The findings do not yet have any real relevance for women, and it is premature to recommend screening women for these gene variants, at least until the scientific community has further combed through the genome-wide findings and found all the variants that are associated with increased risk.

In a third study, a team at deCODE Genetics, the University of Nijmegen in the Netherlands, and elsewhere studied 22,000 people to find two other gene mutations associated with breast cancer. One is also near TNRC9. deCODE estimates that these two variants are contributing factors in 25 % of breast cancer cases in women of European origin.

6.4.2.2 Circulating Nucleic Acid Biomarkers of Breast Cancer

CNA isolated from serum or plasma is increasingly recognized as biomarkers for cancers. Recently developed next-generation sequencing provides high numbers of DNA sequences to detect the trace amounts of unique serum biomarkers associated with breast carcinoma. Serum CNA of women with ductal carcinoma was extracted and sequenced on a 454/Roche high-throughput GS-FLX platform and compared with healthy controls and patients with other medical conditions (Beck et al. 2010). Breast cancer was accurately detected at a diagnostic specificity level of 95 % with a calculated sensitivity of 90 %. Identification of specific breast cancer-related CNA sequences provides the basis for the development of a serum-based routine laboratory test for breast cancer screening and monitoring. This is in development by Chronix Biomedical.

6.4.2.3 Molecular Diagnostic Tests for Breast Cancer

There are several molecular diagnostic tests for breast cancer. A selection of these is shown in Table 6.7.

BRACAnalysis (*Myriad Genetics Inc*.). This test for hereditary breast and ovarian cancers incorporates thorough full-sequence analysis for gene mutation detection. Myriad and others have discovered and published information on an additional type of mutation, known as a large rearrangement that has not been detectable by commercial DNA sequencing technologies, but only by laborious, manual research-based methods. Such rearrangements are responsible for a small percentage of changes in the two breast cancer genes. Myriad added a panel of five common rearrangements to its BRACAnalysis test, accounting for nearly half of the total occurrence of large rearrangements in the two genes. Because large rearrangements are quite rare, a woman meeting the commonly employed selection criteria for BRACAnalysis has less than 0.5 % risk of carrying one of the large rearrangement mutations. Myriad's BRACAnalysis Rearrangement Test (BART) is an automated molecular diagnostic test in the BRACAnalysis family of products, which detects rare, large rearrangements of the DNA in the BRCA1 and BRCA2 genes and is performed in women with exceptionally high risk who have tested negative for sequence mutations and the common large rearrangements already included in Myriad's test.

Next-generation sequencing-based breast cancer genetic test (*NewGene Inc*.). Unlike Myriad's test, BRACAnalysis, which is PCR based, NewGene's assay uses next-generation sequencing technology that results in faster turnaround times and lower costs compared to other technologies. This will lead to improved access to a breast cancer genetic test with clinical use for patients. The test is based on full gene sequencing of the BRCA1 and BRCA2 genes, so it is not targeting specific mutations. NewGene uses the 454/Roche GS-FLX platform for pyrosequencing. Unlike traditional Sanger sequencing, which involves looking at individual segments of a gene one segment at a time and one patient at a time, pyrosequencing enables the investigation of genes of interest in multiple patients in the same run and with multiple gene fragments in the same run. Thus, NewGene can look at 20,000 fragments in one run in contrast to one fragment per run allowed by Sanger sequencing-based methods. Because each patient requires about 100 fragments to be sequenced, the increase in the number of patients that can be investigated in a single run and the improvement in throughput achieved by this technology are significant. Test results using this technology can be achieved in as little as 4 weeks.

Genetic biomarkers for susceptibility to breast cancer. SEQUENOM Inc. has identified novel genetic biomarkers in four genes for susceptibility to breast cancer. Each gene has forms that increase or decrease the risk for developing breast tumors. The company's data indicate that common combinations of its proprietary breast cancer markers increase the average risk of developing breast cancer by a factor of 2, present in approximately 11 % of the female population. Certain rare combinations are estimated to increase the disease risk by up to a factor of 5. The protective

Table 6.7 Molecular diagnostic tests for breast cancer

Test	Company	Description/technique	Purpose	Specimen	Time
Fluorescent in situ hybridization-based tests (FISH)					
Estrogen receptor (ER)/ progesterone receptor (PR)[a]	Exagen	FISH	Initial diagnosis, guide for therapy and prognosis	Fresh tissue or FFPE	3–4 days
SPOT-Light® HER2[a]	Invitrogen/Life Technologies	Chromogenic in situ hybridization (CISH) Kit	To predict response to trastuzumab treatment	Fresh tissue	3–4 days
TOP2A FISH pharmDx test[a]	Dako	FISH	Guide for therapy and prognosis	Fresh tissue or FFPE	3–4 days
Blood tests for biomarkers					
Biomarker translation test		Detects and quantifies key biomarker levels	For screening/early detection of asymptomatic patients	Blood sample	2 weeks
CellSearch®[a]		Enumerates circulating tumor cells of epithelial origin	Early detection in of asymptomatic patients and prognosis	Blood sample	1 day
Detection of gene mutations in blood					
BRACAnalysis[a]	Myriad Genetics Inc.	Detection of hereditary breast cancer	In asymptomatic women with family history	Blood sample	?
BRCA1/BRCA2 testing: based on next-generation sequencing	NewGene Ltd.	Detection of hereditary breast cancer	In asymptomatic women with family history	Blood sample	4 weeks
BRCA MASTR Dx: multiplex amplification of specific targets for resequencing	Multiplicom	Identifies mutations in the coding regions of the BRCA 1 and 2 genes	In women with increased risk for breast cancer	Blood sample	?
Based on gene expression					
BC-SeraPro	Power3 Medical Products Inc.	Measures DNA methylation patterns of breast epithelial cells via quantitative multiplex methylation-specific PCR	Early detection at asymptomatic stage in high-risk patients	Nipple aspirate fluid (NAF)	?
BCtect®	DiaGenic	Analyzes gene expression signatures from peripheral blood	Early detection in asymptomatic patients	Blood sample	<7 days
Breast Cancer Index℠	bioTheranostics/ bioMérieux	HOXB13:IL17BR gene ratio and five-gene expression index	Prognosis: ER+ node-negative breast cancer	Fresh tissue or FFPE	7–10 days
GeneSearch™[a]	Veridex	Detection of gene expression markers mammaglobin and cytokeratin 19 via qRT-PCR	Detects metastasis in lymph nodes in node-negative, T1–T3 invasive carcinoma	Lymph node biopsy	35–40 min

HERmark™	Monogram Biosciences Inc.	Measures total HER2	Guide for therapy and prognosis	Fresh tissue or FFPE	7 days
IVDMIA: in vitro diagnostic multivariate index assay	Bioarray Therapeutics	Integrates data from gene expression microarrays and bioinformatics	Cost-effective personalized test for early diagnosis	Fresh tissue	?
LightCycler HER2/neu	Roche Diagnostics	Real-time PCR for quantification of HER2/neu gene expression	Guide for therapy and prognosis	Fresh tissue or FFPE	7 days
MammaPrint™[a]	Agendia	Measures a 70-gene expression profile	To predict clinical outcome of breast cancer in stage I/II	Fresh tissue	10 days
MapQuant Dx™ Genomic Grade	QIAGEN Marseille	MapQuant Dx™ Genomic Grade	Tumor grading: low versus high from expression levels of 97 key genes	Fresh tissue or FFPE	<10 days
Oncotype DX®	Genomic Health Inc.	Measures ER, PR, and HER2 gene expression via qRT-PCR	Prognostic and predictive value in stages I and II	Fresh tissue or FFPE	10–14 days
OncoVue® Test	InterGenetics Inc.	Combines the SNP patterns of 117 genes and personal history to predict breast cancer risk	Screening of patients without family history	Cheek cell collection by oral rinse	5 days
Prosigna Breast Cancer Prognostic Gene Signature Assay	NanoString Technologies	Digital readout of expression of 50 genes implicated in the growth and spread of cancer	Score to estimate chance that cancer recur after hormone therapy	Fresh tissue or FFPE	?
Based on IHC					
ForeCYTE	Atossa Genetics Inc.	IHC of cells and biomarkers in NAF	Risk of breast cancer in women 18–65 years of age	NAF	5 min
Insight® Dx	Clarient Inc.	Combines traditional risk factors with seven key molecular biomarkers	Prognostic test for early-stage, hormone receptor-positive breast cancer	Fresh tissue or formalin-fixed paraffin-embedded (FFPE)	3–4 days
Combination of clinical risk and genetic biomarkers					
BREVAGen test	Genetic Technologies	Seven SNPs and risk factors	Provides a 5-year and lifetime risk of breast cancer	Blood	?

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[a]Approved by the FDA

forms of the genes are present in approximately 13 % of the female population and are associated with a fivefold decreased risk of developing breast cancer compared to the general population. This marker panel was identified in SEQUENOM's discovery genetic program using unrelated patient and control subjects of European descent. The company has replicated the initial association for these markers in an independent Australian cohort. Knowledge of genetic risk can allow for early intervention or prophylactic treatment options to offset that risk.

Staging of breast cancer. Although distinct pathological stages of breast cancer have been described, the molecular differences among these stages are largely unknown. In situ gene expression profiles of the premalignant, preinvasive, and invasive stages of human breast cancer—generated through the combined use of LCM and DNA microarrays—reveal extensive similarities at the transcriptome level among the distinct stages of progression and suggest that gene expression alterations that confer the potential for invasive growth are already present in the preinvasive stages. In contrast to tumor stage, different tumor grades are associated with distinct gene expression signatures. Furthermore, a subset of genes associated with high tumor grade is quantitatively correlated with the transition from preinvasive to invasive growth.

Gene expression profiling of breast cancer. Studies of primary breast tumors performed by different laboratories have resulted in the identification of a number of distinct prognostic profiles, or gene sets. A study has compared the predictions derived from these gene sets for individual samples (Fan et al. 2006). Even though different gene sets were used for determining prognosis in patients with breast cancer, four of the five tested showed significant agreement in the outcome predictions for individual patients and are probably tracking a common set of biological phenotypes. NanoString's Prosigna Breast Cancer Prognostic Gene Signature Assay (marketed in EU) provides a digital readout on the expression of 50 genes that are implicated in the growth and spread of cancer as compared to Genomic Health's 21-gene Oncotype that is used mostly in the USA.

ForeCYTE Breast Health test (*Atossa Genetics Inc*.). ForeCYTE, a "Pap test for breast cancer," is designed to collect nipple aspirate fluid (NAF), which contains cells from the lining of the milk ducts and the lobules, where ~99 % of all breast cancers originate. NAF is analyzed to determine whether the cells are normal, atypical (premalignant), or malignant, using a patented, multiplex, IHC procedure. This test is intended as an adjunct to mammography for women aged 50–75 and for younger women between 20 and 49 years of age for whom screening mammography is not recommended due to its poor sensitivity in this age group and potential radiation safety issues. The sensitivity and specificity for a woman with atypical cells in NAF being diagnosed with breast cancer within a 2-year period are 92 % and 85 %, respectively. The negative predictive value, i.e., the likelihood of a woman with a normal ForeCYTE Breast Health test not being diagnosed with breast cancer in the following 2-year period, is 99.7 %.

Ethical issues of genetic testing for breast cancer. Several ethical issues are raised by testing for genetic predisposition to cancer in view of the psychological and

social consequences of the test results. According to the guidelines set by the American Cancer Society, testing of individuals for a mutation linked to susceptibility for cancer should be done if the probability of detecting it is 10 % or more. Myriad has calculated that this threshold has been reached for BRCA1 in the case of women whose families have had two cases of breast cancer before the age of 50. The chance of detecting the mutation rises to 30 % in women who have one relative with breast cancer and one with ovarian cancer. Mutations are identified in 13 % of women who report a family history of only a single first-degree or second-degree relative with early-onset breast cancer or ovarian cancer, which means that they are more than 100 times more likely to carry a mutation than a woman in the general population. This shows that cancer gene test is valuable even for women with just one relative with early onset of those cancers.

Concluding remarks. Genetic testing for inherited mutations in BRCA1 and BRCA2 has become integral to the care of women with a severe family history of breast or ovarian cancer, but an unknown number of patients receive negative (i.e., wild-type) results when they actually carry a pathogenic BRCA1 or BRCA2 mutation. The mutational spectra of BRCA1 and BRCA2 include many high-penetrance, individually rare genomic rearrangements. Among patients with breast cancer and severe family histories of cancer who test negative (wild type) for BRCA1 and BRCA2, approximately 12 % can be expected to carry a large genomic deletion or duplication in one of these genes, and approximately 5 % can be expected to carry a mutation in CHEK2 or TP53 (Walsh et al. 2006). It is recommended that effective methods for identifying these mutations should be made available to women at high risk.

6.4.2.4 Mouse ESC-Based Assays to Evaluate Mutations in BRCA2

BRCA1 and BRCA2 sequence variants do not all confer the same risk for breast cancer. Some are extremely deleterious, while others are neutral or low risk. Distinguishing between these extremes can be difficult, since mutations are often identified through genetic tests of families with a history of breast and ovarian cancer. Segregation analysis in cancer-afflicted families provides the most reliable information to distinguish between deleterious and neutral alterations identified in BRCA1 or BRCA2. Nevertheless, there is a need for a functional assay to classify variants for which such information is not available, because most mutations are rare, and familial data are often insufficient. That also makes it difficult to interpret BRCA1/BRCA2 genetic tests for those carrying unclassified or minor mutations such as the 1,900 known BRCA1 or BRCA2 variations that do not disrupt the gene product in an obvious way but may still affect gene function.

Scientists at the National Cancer Institute (NCI) in the USA and the Sanger Institute in the UK have used mouse ESCs to evaluate the functional implications of 17 BRCA2 sequence variants, and their assay could effectively and reliably categorize risky BRCA2 mutations, which can dramatically increase an individual's risk of breast and ovarian cancer (Kuznetsov et al. 2008). Overall, they classified eight of the 17 mutations as deleterious. The remaining nine appear to be either neutral or

low risk. The researchers exploited the fact that mouse ESCs need a functional copy of BRCA2 to survive. They generated mouse ESCs that were missing one copy of BRCA2 and one conditional BRCA2 allele. Then, they added BACs containing individual human BRCA2 variants, inactivated the lone copy of mouse BRCA2, and looked at whether the cells could survive by relying on that human BRCA2 gene. Variants that could not rescue the BRCA2 function in these mouse ESCs were classified as deleterious mutations, while those that could were considered neutral mutations. This assay is likely to improve the understanding of unclassified mutations because it enables analysis of all types of BRCA2 mutations. However, it will take time before the assay can be used as a clinical tool. Until the assay is fully validated, caution must be exercised when using these data to make clinical decisions. The scientists are interested in collaborating with commercial organizations to develop the diagnostic test. They are optimistic that such assays will eventually be used to characterize not only BRCA2 variants but also variants in other disease-associated genes.

6.4.2.5 Genomic Profiles of Breast Cancer

Large-scale DNA alterations in cancer cells—rearrangements, deletions, and duplications—may assist in the proliferation and progression of the disease. The use of a high-resolution genomic profiling technique called ROMA (Representational Oligonucleotide Microarray Analysis) for the analysis of genomic alterations in breast tumors has enabled identification of three distinct patterns of genomic variation, one of which termed “firestorms” may be predictive of aggressive disease progression and short survival. “Firestorms” are violent genomic disruptions that lead to destructive forms of breast cancer, even when the rest of the genome is relatively quiet. “Firestorms” were found in 25 % of the breast cancer samples and were associated with negative clinical outcomes. In these cases, there were genomic amplification–tight chromosomal clusters where DNA segments had undergone multiple rounds of breakage, copying, and rejoining in a concerted manner. The amplifications were generally limited to single chromosomal arms and were flanked by broad segments of low copy number duplications and deletions.

Another complex genomic profile, called “sawtooth,” was present in 5 % of breast cancer samples. It was characterized by narrow, low copy number deletions and duplications that were evenly distributed across the chromosomes. The “simplex” profile, affecting 60 % of the tumor samples, exhibited broad genomic duplications and deletions that only affected a single chromosomal arm. The remaining 10 % of the samples exhibited a “flat” profile, reflecting normal levels of copy number variation in the genome.

Gene expression arrays have generated molecular predictors of relapse and drug sensitivity in breast cancer. Exons differently expressed in malignant and benign breast lesions have been used to generate a molecular classifier for breast cancer diagnosis (Andre et al. 2009). Breast samples were obtained by fine needle aspiration (FNA). cDNA was hybridized on splice array, and the nearest centroid

prediction rule was developed to classify lesions as malignant or benign on a training set, and its performance was assessed on an independent validation set. A two-way ANOVA model identified probe sets with differential expression in malignant and benign lesions while adjusting for scan dates. This signature accurately classified all samples with 100 % accuracy. Genes involved in spliceosome assembly were significantly overexpressed in malignant disease. Many exons are differently expressed by breast cancer and benign lesions, and alternative transcripts contribute to the molecular characteristics of breast malignancy. Development of molecular classifiers for breast cancer diagnosis with FNA should be possible. Exonhit is developing such a test.

6.4.2.6 Role of Molecular Diagnostics in Management of Breast Cancer

The information provided by a personal genetic test might be of real value in identifying the woman whose risk for breast cancer or other cancers is likely to be amplified by oral contraceptives. Depending on the mutation, oral contraceptives can increase the risk of breast cancer and may also fail to protect against ovarian cancer. Thus, a positive test for certain genetic mutations means that the strategy of using oral contraceptives to reduce the risk of ovarian cancer should be abandoned. In contrast, a woman worried about ovarian cancer who does not have one of these hereditary contraindications could then take oral contraceptives without danger of precipitating a known hereditary breast cancer.

The affected women also have the option for prophylactic breast removal, which reduces the breast cancer risk by >85 %. Women with a family history of breast cancer that have prophylactic mastectomy show 90 % reduction in the number of breast cancers in high-risk women and BRCA mutation carriers. Chemoprevention with tamoxifen or other agents is another option. The goal is to make chemoprevention as effective as prophylactic mastectomy. More than 20 drugs are available to treat breast cancer. The researchers are now investigating how each tumor type responds to these drugs to help determine the best treatment for each.

There is evidence that some of the gene mutations in breast cancer are relevant to treatment. The HER2 gene also known in avian species as c-erbB-2 (avian *er*ythro*b*lastic leukemia viral oncogene homolog 2) or in the rat as neu (*neu*roblastoma glioblastoma oncogene) is amplified in 20–30 % of breast cancers. HER2 gene amplification and HER2 overexpression occur early in the development of breast cancers and are found in a high proportion of ductal carcinomas in situ (DCIS), noninvasive cancers that generally do not give rise to metastases. In DCIS, HER2 overexpression is found specifically in poorly histologically differentiated disease and not in well-differentiated cancers. Various methods have been used to analyze the HER2 status of a tumor:

- IHC: protein expression levels
- ELISA: shedding of HER2 receptor
- FISH: HER2 gene amplification

- qPCR: HER2 gene amplification
- Quantitative RT-PCR: mRNA expression level

In practice, IHC is the most frequently used method. However, it is recommended that all specimens with weakly positive IHC (+2 HercepTest result) be evaluated by FISH for HER2/neu gene amplification. The results of both assays should be considered before making a decision to recommend anti-HER2 therapy. The LightCycler™ PCR assay (Roche) has now been developed specifically to assess HER2 gene amplification. The advantages are:

- It is accurate for determining HER2 gene amplification and correlates well with FISH: 85 % sensitivity and 95 % specificity.
- It is a rapid screening method with up to 30 samples per run.
- The kit uses a reference sequence on chromosome 17 so that a correct data interpretation should be possible in polysomic cases.

One limitation of LightCycler PCR is that it does not give histopathological assignment. Microdissection may be required in critical cases. Current methods for checking HER2 are problematic because of issues with intra- and interlaboratory reproducibility and preanalytic variables, such as fixation time. In addition, the commonly used HER2/chromosome 17 ratio presumes that chromosome 17 polysomy is present when the centromere is amplified, even though analysis of the rest of the chromosome is not included in the assay. In one study, 97 frozen samples of invasive lobular and invasive ductal carcinoma, with known ICH and FISH results for HER2, were analyzed by aCGH to a commercially available bacterial artificial chromosome whole-genome array containing 99 probes targeted to chromosome 17 and the HER2/TOP2 amplicon (Yeh et al. 2009). Results were 97 % concordant for HER2 status, meeting the College of American Pathologists/American Society of Clinical Oncology's validation requirements for HER2 testing. No case of complete polysomy 17 was detected even though multiple breast cancer cases showed polysomies of other chromosomes. Therefore, aCGH is an accurate and objective DNA-based alternative for clinical evaluation of HER2 gene copy number, and that polysomy 17 is a rare event in breast cancer. It is commercially available as HER2 PRO (CombiMatrix Molecular Diagnostics).

Qualitative real-time PCR (*real-time-qPCR assays*) *assays*. These have been used to risk-stratify breast cancers based on biological "intrinsic" subtypes and proliferation. Real-time qPCR is attractive for clinical use because it is fast, reproducible, tissue-sparing, quantitative, and automatable and can be performed from archived (formalin-fixed, paraffin-embedded tissue) samples. The benefit of using real-time qPCR for cancer diagnostics is that new biomarkers can be readily validated and implemented, making tests expandable and/or tailored to the individual. For instance, the proliferation metagene could be used within the context of the intrinsic subtypes or used as an ancillary test in breast cancer and other tumor types where an objective and quantitative measure of grade is important for risk stratification. As more prognostic and predictive signatures are discovered from microarray, it should be possible to build

on the current biological classification and develop customized assays for each tumor subtype. This approach enables the important clinical distinction between ER-positive and ER-negative tumors and identifies additional subtypes that have prognostic value. The proliferation metagene offers an objective and quantitative measurement for grade and adds significant prognostic information to the biological subtypes. It is a robust predictor of survival across all breast cancer patients and is particularly important for prognosis in luminal A (ER-positive) breast cancers, which have a worse outcome than expected when proliferation is high. This supports previous findings that a genomic signature of proliferation is important for predicting relapse in breast cancer, especially in ER-positive patients.

A study has compared qRT-PCR results for the assessment of mRNA levels of ERα, PgR, and the members of the human EGFR family, HER1, HER2, HER3, and HER4 (Labuhn et al. 2006). The results were obtained in two independent laboratories using two different methods, SYBR Green I and TaqMan probes, and different primers. By linear regression, a good concordance was demonstrated for all six biomarkers. The quantitative mRNA expression levels of ERα, PgR, and HER2 also strongly correlated with the respective quantitative protein expression levels prospectively detected by EIA in both laboratories. In addition, HER2 mRNA expression levels correlated well with gene amplification detected by FISH in the same biopsies. These results indicate that both qRT-PCR methods were robust and sensitive tools for routine diagnostics and consistent with standard methods. The simultaneous assessment of several biomarkers is fast as well as labor effective and optimizes the clinical decision-making process in breast cancer tissue and/or core biopsies.

SPOT-Light® HER2 CISH Kit (*Invitrogen/Life Technologies*). This test, which is approved by the FDA, is based on a technology called chromogenic in situ hybridization (CISH). The test uses a DNA probe for the HER2 gene and predicts whether a breast cancer patient is a candidate for trastuzumab treatment. Current medical practice requires that all patients who are considered for trastuzumab treatment be tested for HER2 amplification or overexpression. CISH test results are visualized under a standard bright-field microscope, as opposed to FISH tests, in which the results must be visualized using a fluorescent microscope. This specialized microscope frequently requires that the analysis is done at a reference lab. In addition, HER2 CISH test results are quantifiable, removing the subjectivity inherent in tests based on IHC interpretation schemes.

Multiplex ligation-dependent probe amplification (*MLPA*). Next to IHC to evaluate HER2 protein overexpression, a second-line gene amplification test is generally deemed necessary for cases with equivocal protein expression. MLPA, a PCR-based test, is a simple and quick method to assess HER-2/neu gene amplification status in invasive breast cancer. MLPA was previously shown to correlate well with IHC and ISH, but a low tumor percentage in the tissue tested could negatively affect the accuracy of MLPA results. Laser capture or manual microdissection increase the dynamic range of MLPA copy number ratios, which is a technical advantage (Moelans et al. 2009).

GeneSearch™ (Veridex) breast sentinel lymph node intraoperative assay. This FDA-approved RT-PCR breast cancer assay on tissue extracted from a sentinel lymph node detects genes, indicating lymph node metastasis of breast cells. Conducted on Cepheid's SmartCycler System, the test can generate results in 35–40 min compared with the 2 or 3 days for standard tissue pathology. GeneSearch enables surgeons to test up to 50 % of the sentinel node, as opposed to only 5 % typically examined under a microscope. The test, which correctly identified 95.6 % of patients who had metastases in their lymph nodes in clinical trials of 300 women, also can be used to make real-time decisions in the operating room about whether to remove a lymph node and could prevent the need for a second operation in ~5,200 breast cancer patients/year in the USA.

Gene expression profiling. Gene expression profiling with the use of DNA microarrays enables measurement of thousands of mRNA transcripts in a single experiment. These are being used to develop new prognostic and predictive tests for breast cancer and might be used at the same time to confirm ER status and ERBB2 status. Gene expression data of breast cancer samples were used to assess the correlation between ER and ERBB2 mRNA and clinical status of these genes as established by IHC or FISH or both (Gong et al. 2007). Amounts of ESR1 and ERBB2 mRNA, as measured by the Affymetrix U133A GeneChip, reliably and reproducibly established ER status and ERBB2 status, respectively. The gene expression tests are 90 % accurate for both receptors, which make them comparable to, if not better than, existing pathology tests. This is one important step toward personalized diagnosis and treatment planning based on an integrated genomic test of an individual tumor.

Resistance to treatment with endocrine therapy occurs in ~50 % of breast cancer patients. The transcription factor PBX1, a known notch target gene, is required for the growth of endocrine therapy-resistant breast cancer cells. The notch pathway is overactivated in resistant breast cancer cells, whereas classical ERα signaling is epigenetically disengaged. Blocking of notch signaling abrogates growth of resistant breast cancer cells. A gene expression signature based on notch–PBX1 activity can determine if breast cancer patients are responsive or not to endocrine therapy (Magnani et al. 2013).

Results of gene expression studies have confirmed that breast cancer is not a single disease with variable morphologic features and biomarkers but, rather, a group of molecularly distinct neoplastic disorders. This forms the basis of molecular classification of breast cancer. Profiling results also support the hypothesis that ER-negative and ER-positive breast cancers originate from distinct cell types and point to biological processes that govern metastatic progression. Moreover, such profiling has uncovered molecular signatures that could determine response to chemotherapy and influence clinical care of patients with breast cancer (Sotiriou and Pusztai 2009).

Unbiased NGS studies have identified several recurrently mutated genes in breast cancer that represent putative novel therapeutic targets (Russnes et al. 2011). PI3KCA was found as one of the most frequently mutated genes in breast and other cancer types. Therapeutic targeting of the PI3K/AKT signaling pathway has been a major focus of several drug companies, leading to the development and clinical

testing of several PI3K and AKT inhibitors. Upregulation of phosphorylated HER3 and partial recovery of phospho-AKT has been observed following XL147 treatment, leading to incomplete suppression of tumor cell growth (Chakrabarty et al. 2012). Based on follow-up experiments, these authors demonstrated that the combined inhibition of HER2 and PI3K leads to synergistic effects and more efficient eradication of the tumors. These results are an example for the complexity of signaling pathways in cancer cells complicated by multiple layers of feedback inhibition.

PAM50 gene signature test (NanoString Technologies). The subtype classification and prognostic score generated by the PAM50 gene signature provides information about the fundamental biology of breast cancer that other available diagnostic tests cannot. Intrinsic subtyping, which can be achieved with PAM50, is useful not only for prognostic applications but also for predicting response to specific chemotherapies. The main subtypes are ER-negative tumors, basal-like and HER2, and two subtypes of ER-positive tumors, luminal A and luminal B. The intrinsic subtypes have been correlated with a number of important outcomes that can enable major therapeutic choices, not just for women who are ER positive and node negative the way some of the existing assays in the market today do but for women who have later stages of breast cancer who are node positive and who are ER negative. PAM50 can guide clinicians in treating all breast cancer patients, and not just a subset. Additionally, the chemistry used for the platform is particularly compatible with FFPE tissue, which is the most common kind of solid tumor tissue sample used for clinical research. In December 2012, results were released from the second clinical validation study of PAM50 gene expression signature, which evaluated samples from more than 1,400 patients enrolled in the Austrian Breast & Colorectal Cancer Study Group 8 trial. The study met its primary and secondary objectives and demonstrated the ability of the PAM50 test to indicate the risk of distant recurrence in postmenopausal women with hormone receptor-positive early-stage breast cancer treated with endocrine therapy alone.

FAST. This combines laser techniques with a whisk-broom bundle of fiber-optic threads, enabling accurate detection of traveling cancer cells, at a much faster pace than current screening allows. The approach also employs a digital microscope to further home in on the pinpointed cancer cells. FAST works by an ethereal method called "collecting the light." The combination of the FAST cytometer and the digital microscope can spot 98 % of the traveling cancer cells in a sample. And it produces a false positive fewer than three times in a million tries—compared with a hundred false positives in a million tries for an automated digital microscope alone—the current most accurate method. FAST cytometer has been tested on blood samples from patients. The system someday could be used alongside mammograms for better breast cancer screening.

Molecular imaging. Over the past few years, MRI has been making an increasingly large contribution to the screening, staging, and follow-up of patients with breast cancer. MRI can be an important supplementary study, but its exact role still needs to be defined. The goal of molecular imaging for breast cancer is to be able to accurately diagnose when the tumor mass has approximately 100–1,000 cells, as opposed

to the current techniques like mammography, which require more than a million cells for accurate clinical diagnosis. This refinement in molecular imaging is feasible with the use of nanobiotechnology. Early diagnosis improves the chances for cure.

Use of PET to determine response to chemotherapy. In patients with metastatic breast cancer, sequential ^{18}F-FDG PET enables prediction of response to treatment after the first cycle of chemotherapy. The use of ^{18}F-FDG PET as a surrogate endpoint for monitoring therapy response offers improved patient care by individualizing treatment and avoiding ineffective chemotherapy.

Prediction of response to chemotherapy by intrinsic subtypes. A 50-gene subtype predictor was developed using microarray and quantitative RT-PCR to improve on current standards for breast cancer prognosis and prediction of chemotherapy (Parker et al. 2009). It incorporates the gene expression-based intrinsic subtypes luminal A, luminal B, and HER2-enriched, which are generally considered types with a poor prognosis. Breast cancer experts also typically identify a fifth breast cancer type known as normal-like. The 50-gene set also recognizes the normal-like type, but instead of being a fifth type of breast cancer, the normal-like classification is an indicator that a sample contains insufficient tumor cells to make a molecular diagnosis and that a new sample needs to be taken. The genetic test was highly sensitive and very predictive for chemotherapy response. The test was more predictive than typically used clinical molecular markers such as ER status, progesterone receptor (PR) status, or HER2 gene expression status. Luminal A was found to be not sensitive to the chemotherapy, suggesting that patients with this good-prognosis type can forgo chemotherapy in favor of hormone-based therapy. Among the poor-prognosis tumor types, basal-like breast cancer was the most sensitive to the chemotherapy and luminal B the least.

Diagnosis by intrinsic subtype adds significant prognostic and predictive information to standard parameters for patients with breast cancer. The prognostic properties of the continuous risk score will be of value for the personalized management of node-negative breast cancers. The subtypes and risk score can also be used to assess the likelihood of efficacy from neoadjuvant chemotherapy. This new genomic test is broadly applicable for all women diagnosed with breast cancer. Their 50-gene set can be assayed in preserved tumor samples left over from standard diagnostic procedures, so that tumor samples from breast cancer cases going back a decade or more can be studied. Since the patients in these cases have already been treated, the researchers can quickly discover how well various therapies worked for each breast cancer type.

Measurement of ER mRNA to predict response to tamoxifen. Quantification of mRNA has historically been done by RT-PCR. A robust method of detection of mRNA utilizing ISH has been described that is linear and shows high specificity with low background. AQUA method of quantitative immunofluorescence (QIF) has been tested for measuring mRNA in situ using ESR1 alpha gene in breast cancer to determine its predictive value compared to ER protein (Bordeaux et al. 2012). mRNA for ER (ESR1) and ubiquitin C (UbC) were visualized using RNAscope

probes, and levels were quantified by quantitative ISH (qISH) on two Yale breast cancer cohorts on tissue microarrays. ESR1 levels were compared to ER protein levels measured by QIF using the SP1 antibody. Results showed that ESR1 mRNA is reproducibly and specifically measurable by qISH on tissue collected from 1993 or later. ESR1 levels were correlated to ER protein levels in a nonlinear manner on two Yale cohorts. High levels of ESR1 were found to be predictive of response to tamoxifen in a manner different from value of ER.

6.4.2.7 Tests for Prognosis of Breast Cancer

Prognostic testing of all patients prior to treatment aligns with standard medical practice to distinguish patients by hormone status This information can also enable pharmaceutical companies to clearly define patient stratification that improves clinical trial timelines and outcomes.

Exagen'*s breast cancer prognostic biomarker assays*. These are the first and only tests to enable specific testing for hormone receptor (including ER and PR)-positive and for hormone receptor-negative patients using an improved FISH assay. These prognostic tests separate patients with good prognosis from those with poor prognosis by testing each patient's tumor tissue to detect changes in DNA (e.g., gene copy number) in order to directly reflect changes in the tumor. Exagen's prognostic tests are uniquely developed as separate sets of DNA markers to identify prognosis in hormone-positive and hormone-negative patients, respectively. Both biomarker sets represent the first prognostic tests that can be used by any FISH testing laboratory, enabling fit of this testing approach with standard hormone testing prior to treatment. Exagen's small, prognostic marker sets combine to form a testing panel that differs from other existing sets of 20–70-gene markers by enabling:

- Use of improved FISH technology with a small (3–5) number of probes to fit with current laboratory testing practices and equipment
- Testing of all breast cancer patients to provide additional prognostic information based on hormone receptor status (including ER and PR) prior to treatment
- Detection and visualization of tumor-based cellular changes to define only those DNA changes that are specific to tumor tissue

Prognostic gene biomarkers of breast cancer. Three genes, homeobox 13 (HOXB13), interleukin-17B receptor (IL17BR) and CHDH, and the HOXB13:IL17BR ratio index in particular, strongly predict clinical outcome in breast cancer patients receiving tamoxifen monotherapy. A tumor bank study demonstrated that HOXB13:IL17BR index is a strong independent prognostic factor for ER+ node-negative patients irrespective of tamoxifen therapy (Ma et al. 2006). As a result of this study, these two biomarkers serve as the foundation of the Breast Cancer Index^{SM} (bioTheranostics/bioMérieux), which predicts breast cancer recurrence, resulting in more appropriate treatment decisions.

Activity of a gene, Dachshund (DACH1), which normally regulates eye development and development of other tissues, commandeers cancer-causing genes and returns them to normal. DACH1 inhibits the expression of the cyclin D1 gene, an oncogene that is overexpressed in about half of all breast cancers. Analysis of over 2,000 breast cancer patients has demonstrated that DACH1 correlates with tumor size, stage, and metastasis, with its expression greatly reduced in metastatic breast cancer cells, but increased nuclear DACH1 expression predicts improved patient survival (Wu et al. 2006a). The average survival was almost 40 months longer in women in whom their breast cancer continued to express DACH1. DACH1 gene reverts the cancerous phenotype, thus turning the cell back to a premalignant state, and it could be used as a prognostic marker for breast cancer. Other cell fate-determining genes are being examined in an attempt to identify new therapeutics for breast cancer and metastasis.

Multigene expression prognostic constellation (*Celera*). The prognostic constellation provides information that is distinct from that predicted by routine clinical assessment tools, such as tumor grade, and can quantify risk for metastasis for variable time periods rather than only categorically for 5 or 10 years. A previously developed 14-gene metastasis score that predicts distant metastasis in breast cancer research subjects without systemic treatment has now been applied to tamoxifen-treated research subjects. Many of the genes in this constellation are involved in the p53 and TNF signaling pathways and are implicated in cancer proliferation. The absence of the ER gene in the constellation increases the confidence that this information complements routinely assayed ER levels determined by IHC. The test can be used as a predictor of distant metastasis in tamoxifen-treated breast cancer patients. A key finding is the calculation of a metastasis score for breast cancer that predicts a 3.5-fold difference in risk between the 20 % of women at highest risk and the 20 % of women at lowest risk.

MapQuant Dx™ Genomic Grade™ (*QIAGEN Marseille*). This is a genomic index that can capture proliferation in most tumors and shows superior prognostic and classification value over conventional grading methods. Genomic Grade™ represents the common core feature of the main breast cancer prognostic genomic signatures published worldwide. QIAGEN Marseille has signed a license agreement with Université Libre de Bruxelles of Belgium, where Genomic Grade™ was developed, for exclusive worldwide rights to the test. The use of Genomic Grade™ can identify two clinically distinct ER-positive molecular subtypes in a simple and highly reproducible manner across multiple datasets (Loi et al. 2007). This study emphasizes the important role of proliferation-related genes in predicting prognosis in ER-positive breast cancer.

Insight® Dx Breast Cancer Profile (*Clarient Inc*.). This has been clinically validated as a prognostic test for women with early-stage, hormone receptor-positive breast cancer. It combines three traditional pathology staging risk factors—tumor size, tumor grade, and lymph node status—with seven key molecular biomarkers, which include ER, PR, HER2, EGFR, bcl2, p53, and myc. The information is then

combined with a proprietary algorithm to produce a risk score that assists pathologists and oncologists in clinical decision-making. Clarient conducted an independent study using a set of breast cancer patients from the Royal Perth Hospital in Western Australia to clinically validate the Clarient Insight Dx Breast Cancer Profile. The algorithm demonstrated an accurate, actionable risk recurrence score. In the study, high- and low-risk patients were identified using the Clarient Insight Dx Breast Cancer Profile. The low-risk group had only a 3 % recurrence rate 10 years after surgery. This is equivalent to a negative predictive value of about 97 %, and the corresponding positive predictive value was 39 %. Further details can be seen on the following web site: http://www.clarientinc.com/insightdx.

TaqMan noncoding RNA assays (*Life Technologies*). These assays have helped to uncover regulatory roles of noncoding RNAs in breast cancer. Long intervening noncoding RNAs (lincRNAs) in the HOX loci become systematically dysregulated during breast cancer progression. The lincRNA termed HOTAIR is increased in expression in primary breast tumors and metastases, and HOTAIR expression level in primary tumors is a powerful predictor of eventual metastasis and death (Gupta et al. 2010). Enforced expression of HOTAIR in epithelial cancer cells induced genome-wide retargeting of Polycomb repressive complex 2 (PRC2) to an occupancy pattern more resembling embryonic fibroblasts, leading to altered histone H3 lysine 27 methylation, gene expression, and increased cancer invasiveness and metastasis in a manner dependent on PRC2. Conversely, loss of HOTAIR can inhibit cancer invasiveness, particularly in cells that possess excessive PRC2 activity. TaqMan noncoding RNA assays can accurately measure expression levels of this molecular biomarker in different breast cancer samples.

6.4.3 Cervical Cancer

Cancer of the cervix is the second most common cancer in women. The mortality rates of cervical cancer could be drastically reduced by the implementation of population-wide cytological screening test. Screening for cervical intraepithelial neoplasia (CIN) is usually performed by Pap smear or cervicovaginal lavage. Identification of women with abnormal cervical smears permits early treatment of lesions, but the high rate of false-positive and false-negative results is a cause for concern. The oncogenic HPV is the causal factor in the development of cervical cancer. The detection of the viral infection enables the identification of patients at risk, but 5–30 % of the normal female population harbors these viruses, and only very few of these develop clinically relevant lesions.

Molecular diagnosis of HPV using Digene Corp.'s Hybrid Capture (HC)2 assay has been described in Chap. 6. HC2 assay has a greater sensitivity to detect CIN grade 3 or higher, and its specificity is comparable to an additional cytological test indicating atypical squamous cells of undetermined significance or a more advanced lesion. Testing for high-risk HPV with the HC2 test is useful in the detection of CIN

grade 2/3 in low-grade CIN groups and in the selection of patients for colposcopy. Quest Labs now provides HC2 test as a primary tool to detect cervical cancer along with Pap smears rather than as a secondary test. A growing body of data now demonstrates the ability of HPV testing to identify women at high risk of cervical cancer more accurately than the most advanced type of Pap. Some experts recommend that HPV test should replace Pap as the first-line tool for cervical cancer screening, particularly in low-resource countries in the developing world. HPV screening that distinguishes HPV16 and HPV18 from other oncogenic HPV types may identify women at the greatest risk of developing cervical cancer.

A study found that nearly half of the women who had been called HPV negative by the HC2 test had detectable levels of HPV DNA by the PCR-MS method (Patel et al. 2009). Because the PCR-MS method also measures the quantity of HPV relative to the number of human cells in the sample, or "viral load," ~15 % of these samples had HPV loads comparable to the HPV loads found in women called HPV positive by the HC2 test. While further work to define clinically meaningful HPV detection thresholds is required, these results suggest that a type-specific, qPCR-MS-based test may be an important advance in the early detection of cervical cancer. AttoSense HPV assay (SEQUENOM) detects, identifies, and quantifies each of 15 HPV types associated with cervical cancer in a single reaction. Preliminary research studies show that the test can measure and reproducibly detect as few as 50 DNA copies for each of the 15 HPV genotypes in liquid cytology samples from cervical smears and tissue biopsy samples. The AttoSense test is currently available for research use only.

In advanced preneoplastic lesions, HPV genomes are often integrated into cellular chromosomes. This leads to enhanced expression of the viral oncogenes. The detection of specific viral mRNA transcripts derived from integrated HPV genomes enables the identification of preneoplastic lesions with a particularly high risk for progression to invasive cancers (APOT assay). These findings will enable establishment of highly sensitive but specific and cost-efficient new cancer early detection assays.

The activity of two viral oncogenes E6 and E7 initiates, in a long-term process, neoplastic transformation in few of the HPV-harboring cells. As consequence of the expression of E7, a cellular marker protein p16 is increasingly expressed in dysplastic cells. MAbs directed against p16 enable specific identification of dysplastic cells and derived invasive cancers in histological slides and in cytology smears (CINtec Assay, MTM Laboratories). CINtec, by correlating the cancer-specific antigen to the histology and cytology, will provide more detailed and precise information for cancer screening and diagnosis to the pathologist. Clinical study data have already shown very promising results in its application for the early detection of cervical cancer. InPath System (Molecular Diagnostics Inc.) uses a specific combination of protein-based biomarkers to illuminate and map abnormal cells as a useful method of screening for cervical cancer.

The Quest Diagnostics Cervical Cancer test is based on the human telomerase RNA component (TERC) gene marker. It helps in the identification of women with unclear Pap smear results, who are at increased risk of developing cervical cancer. Amplification of TERC gene is indicated by an abnormal number of copies of the gene on chromosome arm 3q.

6.4.4 *Colorectal Cancer*

CRC is one of the most common cancers in the world. The lifetime risk of CRC among whites in the Western world is approximately 4 %. The cause of CRC is multifactorial, involving hereditary susceptibility, environmental factors, and somatic genetic changes during tumor progression.

6.4.4.1 ColoVantage CRC Test

ColoVantage CRC test (Quest Diagnostics) detects methylated DNA of the septin 9 gene from a patient's blood sample. In a clinical validation study, ColoVantage identified CRC in 70 % of samples from people diagnosed with CRC and correctly detected the absence of cancer in about 89 % of samples. ColoVantage was approved by the New York State Department of Health in 2011 for use in the state, making it available in all 50 states. It is the first molecular CRC detection method employing venous blood specimen to be approved by the state. The test cannot replace colonoscopy and is not validated as a general screening test, but the test's ability to detect this cancer may persuade nonadherent screening-eligible individuals who receive a positive result to undergo colonoscopy or other evaluation.

6.4.4.2 Detection of Familial Adenomatous Polyposis Coli

Familial adenomatous polyposis coli (APC), characterized by the development of multiple colon polyps in young adults, is an autosomal-dominant disease caused by a germline mutation of the APC gene. Although this disease has been thoroughly studied at the molecular genetic level, the function of APC gene product remains largely unknown. If these patients do not receive a prophylactic colectomy, they develop cancer of the colon by the sixth decade of life.

COLARIS AP® (Myriad Genetics) is a predictive medicine product for risk of hereditary colorectal polyps and cancer. COLARIS AP® detects mutations in the APC and MYH genes, which cause adenomatous polyposis syndromes, including familial adenomatous polyposis (FAP), attenuated familial adenomatous polyposis (AFAP), and MYH-associated polyposis (MAP).

6.4.4.3 Detection of CRC at Precancerous State

Three-dimensional pictures of mitotic spindles in intestinal tissue, generated with state-of-the-art imaging techniques, have revealed that they behave differently in tissue with a single mutation associated with CRC than they do in normal, healthy tissue (Quyn et al. 2010). The alignment of mitotic spindles perpendicular to the apical surface specifically in the stem cell compartments of the intestine correlates with the asymmetric retention of label-retaining DNA. Both the preference for

perpendicular spindle alignment and asymmetric label retention are lost in precancerous tissue heterozygous for the adenomatous polyposis coli tumor suppressor (APC). These data suggest that loss of asymmetric division in stem cells might contribute to the oncogenic effect of APC mutations in gut epithelium. This may have implications in developing methods for identifying precancerous tissue at an early stage, when it still appears normal. It might also be relevant to the types of treatment that should be considered. Studies done by the Mayo Clinic have shown that two precursors of CRC can be detected through noninvasive stool DNA testing: premalignant dysplasia in patients with IBD and serrated polyps.

6.4.4.4 Detection of Circulating Tumor Cells in Colorectal Cancer

Guanylyl cyclase C (GCC), a receptor found on intestinal mucosa cells, may serve as a biomarker of circulating CRC cells. GCC-specific nested RT-PCR can be used to detect CTCs in patients with CRC. Because GCC is ectopically expressed by blood mononuclear cells, a high false-positive rate occurs in healthy volunteers. The current findings point to CD^{34+} cells as the source for ectopic expression. Limiting the amount of RNA analyzed or depleting CD^{34+} cells can improve the false-positive rate.

K-ras oncogenes have been associated with the development of CRC. Microfabrication and operational characteristics of a simple flow-through biochip sensor has been described that is capable of detecting low-abundance point mutations in K-ras oncogenes from genomic DNA, which carry high diagnostic value for CRC. The biochip consists of an allele-specific ligase detection reaction coupled to a universal array for interrogating multiple mutations simultaneously from a clinical sample.

6.4.4.5 Diagnosis of Hereditary Nonpolyposis Colorectal Cancer

Hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) is a familial cancer syndrome characterized by mutations in at least one of six DNA mismatch repair genes: hPMS1, hPMS2, hMSH2, MSH6, hTGFBR2, and hMLH1. From 5 to 10 % of the 150,000 cases of CRC diagnosed each year in the USA are of hereditary type. Identification of DNA microsatellite instability refines the diagnosis of HNPCC, allowing frequent early-onset colonoscopic screening to be restricted to individuals with an especially high risk of this type of cancer. It is possible that a combination of tests for microsatellite instability, allelic loss, p53 mutations, and other genetic alterations in patients with early-stage CRC will define groups of patients who require different adjuvant therapies or no systemic treatment at all.

The identification of mutations in mismatch repair genes of some HNPCC families provides a basis for molecular diagnosis. Two approaches may be used: assessment of microsatellite instability in tumor specimens and mismatch repair gene sequence analysis of nontumor DNA. Clinical laboratories currently use either the protein truncation test or direct sequencing to find mutations in hMSH2 or hMLH1. For families found to carry harmful mutations to hMSH2 and hMLH1, genetic

testing is indisputably useful. Effectiveness of screening with IHC analysis of the mismatch repair proteins is similar to that of the more complex strategy of genotyping for microsatellite instability. COLARIS® (Myriad Genetics), a predictive medicine test for HNPCC, detects disease-causing mutations in three genes, MLH1, MSH2, and MSH6, which are responsible for the majority of HNPCC.

Routine molecular screening of patients with CRC for the Lynch syndrome has identified mutations in patients and their family members that otherwise would not have been detected. Family members with such mutations should receive frequent diagnostic tests for colorectal and related cancers and may wish to consider prophylactic surgery—such as removal of the colon, uterus, or ovaries—to prevent a cancer occurrence. In families where harmful mutations are not discovered, or where mutations are not clearly linked with disease, careful monitoring of health is called for, and frequent diagnostic tests to catch any cancers in their earliest stages, when they are most easily treated.

6.4.4.6 Diagnosis of CRC from DNA in Stools

There are several available methods of detection for CRC; however, few of these approaches have been established as screening methods, either because the preparation procedures are uncomfortable and unpleasant or because of low sensitivity or specificity. A study found that FDA-approved InSure® (Quest Diagnostics), a fecal immunochemical test, detected three times as many early-stage CRCs and nearly two times as many significant (precancerous) adenomas compared to Hemoccult II Sensa (Beckman Coulter), the traditional guaiac fecal occult blood test (Smith et al. 2006). Examination of DNA in stool samples from patients is a promising screening method. Immunocytochemical analysis of minichromosome maintenance proteins in colonocytes retrieved from the fecal surface is also useful for diagnosis of CRC.

DNA has been purified from routinely collected stool samples and screened for APC mutations with the use of digital protein truncation. APC mutations can be detected in a sensitive and specific manner in fecal DNA from patients with relatively early CRC. Cologuard™ (Exact Sciences), a CRC screening test based on stool DNA changes, has demonstrated a sensitivity of 64 % for precancerous lesions and 85 % for cancerous lesions and a specificity of 88 % in a validation study. Quantitative Allele-specific Real-time Target and Signal (QUARTS) amplification assay technology was used to develop Cologuard, as well as the biomarkers contained in the test. The study took three distinct pathways, using four DNA methylation markers, two DNA mutation markers, and a human blood protein biomarker. DeeP-C clinical trial, completed in 2013, evaluated the test for detection of CRC and precancerous polyps. Preliminary results showed that Cologuard has 92 % sensitivity for the detection of CRC and 42 % sensitivity for the detection of precancerous polyps, including 66 % sensitivity for polyps equal to or greater than 2 cm. The test achieved a specificity of 87 % during the trial. Cologuard is an investigational device and is not available for sale in the USA. Additional analysis by the company and review by the FDA are pending.

DNA methylation is a common molecular alteration in CRC cells. MethyLight analysis of fecal DNA enables identification of SFRP2 methylation as a sensitive single DNA-based biomarker of CRC in stool samples with a sensitivity of 90 % and specificity of 77 %. Whether a combination of genetic and epigenetic markers will identify CRC at an early stage remains to be shown.

6.4.4.7 Early Diagnosis of CRC from Blood Samples

BioServe and Phenomenome Discoveries Inc. (PDI) have developed a novel serum-based diagnostic test for the identification of CRC and precancerous states conducive to the development of CRC. BioServe identified a large number of patient tissue and serum samples from its Global Repository exhibiting CRC across a spectrum of stages, as well as matched healthy controls. Using PDI's patented nontargeted metabolomic platform, PDI discovered that a series of novel metabolites were significantly decreased in serum samples collected from CRC patients compared to controls. From these results, PDI developed a 2-m high-throughput screening method capable of simultaneously measuring a key subset of these molecules. The rapid test was found to have a sensitivity of 75 % and specificity of 90 %. Trials in Canada and Japan are evaluating the test's utility as part of a broad-based population screening regimen.

ColoVantage (Quest Diagnostics) is a test for CRC based on detection of methylated septin 9 DNA in blood, which has been shown to have a sensitivity of 70 % and specificity of 90 % for CRC (Weiss and Rösch 2010). It is important for detecting CRC as 30–40 % of persons have small polyps, but <10 % of these become cancerous. Based on stool test, physicians may be sending far too many people in for colonoscopy. The blood-based test has a limitation in that a positive signal could come from cancers anywhere in the body.

ColonSentry (GeneNews) is based on the Sentinel Principle™, which uses blood samples to identify RNA biomarkers for early diagnosis of CRC. It has been developed to provide:

- A simply convenient first step for CRC screening
- High patient acceptance
- To exploit genomics for CRC diagnostics
- To enhance the number of cancers detected by colonoscopy

6.4.4.8 Guanylyl Cyclase C Tests for CRC

GCC is a cell surface molecule found on colorectal cells, both normal and cancerous, but not on any normal cells outside the intestine. GCC receptor provides a superior mechanism for detecting the presence of CRC cells because it relies on ultrasensitive mRNA-based amplification technology rather than other less sensitive and variable detection systems, such as the histopathology. Quantification of GCC mRNA in tissues by RT-PCR employing external calibration standards is analytically robust

and reproducible, with high clinicopathologic sensitivity and specificity. GCC biomarker has shown to be 95–100 % accurate in detecting the spread or recurrence of CRC in lymph nodes or blood. Preliminary results on the GCC lymph node study for staging CRC corroborate the conclusion of earlier studies. DiagnoCure plans to launch Previstage™ GCC as a lymph node test for the staging of CRC.

6.4.4.9 Minimally Invasive Screening for Colorectal Cancer

Colopath®/ColorectAlert™ (Ambrilia Biopharma) is a highly sensitive and minimally invasive screening and monitoring test for CRC. Colopath® screens for a phospholipid analyte (plasmalogen) in rectal mucus of individuals with colorectal pathology, whereas ColorectAlert™ screens for the T antigen, a complex sugar in rectal mucus. The Colopath®/ColorectAlert™ test involves the application of a rectal mucus sample to a test strip, and a positive/negative result is based on a Schiff's aldehyde reaction. Histological examination of colon biopsies has revealed that the colonic lining of Colopath®/ColorectAlert™-positive individuals may have an abnormal appearance characterized by certain cells, which have become distended and swollen. This abnormal appearance may be characteristic of a colon which is predisposed toward pathological changes. Increased monitoring of Colopath®/ColorectAlert™-positive individuals may, therefore, enable earlier detection of CRC. When combined with fecal occult blood test, it is found that over 99 % of patients who are negative for both tests have no cancer. The test is in clinical trials, and the manufacturer plans to apply for approval.

6.4.5 Gastric Cancer

Gastric cancer is the second most common cause of cancer-related death worldwide. The prognosis is poor, with limited treatment options. A better understanding of the basic mechanisms of gastric carcinogenesis would enable the development of general screening strategies and personalized treatment. Serum-based screening of biomarkers is not ready for routine application. Infection with *Helicobacter pylori* represents the principal risk factor for gastric carcinogenesis. Bacterial virulence and host genetic factors contribute to individual susceptibility. Key molecular alterations in gastric carcinogenesis are related to intra- and extracellular cascades that regulate cell proliferation, tumor invasion, and metastatic spread.

6.4.6 Head and Neck Cancer

Cancers of the oral cavity, salivary glands, larynx, and pharynx, collectively referred to as squamous cell carcinomas of the head and neck (HNSCC), are the sixth most

common cancer among men in the developed world. The prognosis of HNSCC patients is still poor, which reflects the fact that although the risk factors for HNSCC are well recognized, very little is known about the molecular mechanisms responsible for this malignancy.

LCM technologies will be helpful in molecular studies aimed at revealing the mechanisms involved in squamous cell carcinogenesis. They are also expected to provide a molecular blueprint for HNSCC, thus helping to identify suitable markers for the early detection of preneoplastic lesions, as well as novel targets for pharmacological intervention in this disease.

6.4.6.1 Nanobiochip Sensor Technique for Analysis of Oral Cancer Biomarkers

The majority of oral squamous cell carcinomas (OSCCs) are preceded by visible changes in the oral mucosa, most often white patches. Although the histological finding of dysplasia in oral white patches signals increased risk of developing OSCC, this may also occur in non-dysplastic lesions. However, no reliable biomarkers exist to predict the occurrence of OSCC in these patients. Biopsies of these lesions have been analyzed with respect to their DNA content. Aberrant DNA content reliably predicts the occurrence of OSCC in patients that otherwise would be regarded as at very low risk, whereas normal DNA content indicates low risk.

A pilot study has described a nanobiochip sensor technique for the analysis of oral cancer biomarkers in exfoliative cytology specimens, targeting both biochemical and morphologic changes associated with early oral tumorigenesis (Weigum et al. 2010). Oral lesions from dental patients, along with normal epithelium from healthy volunteers, were sampled using a noninvasive brush biopsy technique. Specimens were enriched, immunolabeled, and imaged in the nanobiochip sensor according to previously established assays for the EGFR biomarker and cytomorphometry. Four key parameters were significantly elevated in both dysplastic and malignant lesions relative to healthy oral epithelium, including the nuclear area and diameter, the nuclear-to-cytoplasmic ratio, and EGFR biomarker expression. Further examination using logistic regression and receiver operating characteristic curve analyses identified morphologic features as the best predictors of disease individually, whereas a combination of all features further enhanced discrimination of oral cancer and precancerous conditions with high sensitivity and specificity. Further clinical trials are necessary to validate the regression model and evaluate other potential biomarkers. Nanobiochip sensor technique is a promising tool for early detection of oral cancer, which could enhance patient survival.

6.4.6.2 ProteinChip for Diagnosis of Head and Neck Cancer

Using a ProteinChip, tumor and normal tissue from head and neck cancer and microdissected melanoma have been analyzed to determine differentially expressed proteins.

Comparisons of the protein expression patterns from microdissected normal and tumor tissues indicated several differences, highlighting the importance of extremely defined tissue lysates for protein profiling. By applying this fast and powerful ProteinChip array technology, it becomes possible to investigate complex changes at the protein level in cancer associated with tumor development and progression.

6.4.7 Hematological Malignancies

Most of hematological malignancies are leukemias. Conventional methods for the diagnosis of leukemias are blood counts with staining and examination of cells, examination of bone marrow following aspiration, biochemical screening, chromosome analysis of cells to detect dislocations, and immunophenotyping. Molecular diagnostics is now applied for assessment of leukemias as well as well as chronic myeloproliferative disease (CMPD).

6.4.7.1 Chromosome Translocations

Chromosome translocations, which are present in most human leukemias, are widely used by clinicians as diagnostic and prognostic tools. At the molecular level, translocations are especially valuable because they immediately indicate the spot in which to search for a cancer gene. Chromosome breakpoints can now be cloned and sequenced efficiently, and the relevant genes can be rapidly identified. The t(9;22) translocation known as the Philadelphia chromosome was the first tumor-specific cytogenetic marker identified in a human cancer. Its discovery eventually led to the cloning of the BCR–ABL fusion region. The presence of a Philadelphia chromosome confers a poor prognosis in cases of acute lymphoblastic leukemia (ALL). Other translocations have since been identified, including those involving chromosome segment 11q23.

6.4.7.2 Flow Cytometry in Diagnosis of Leukemia

Flow cytometry is a rapid way to measure the characteristics of individual cells. Hematopoietic cells (blood, bone marrow, core biopsies, lymph nodes) are labeled with selective fluorescent antibodies and quantified according to their surface antigens. These fluorescent antibodies bind to specific leukemic cells in a pattern of antigen expression that is used to identify malignant cell types. Panels of antibodies are often used to help define which malignant cell types are present. The following types of leukemias can be identified and characterized through the use of flow cytometry: acute myelogenous leukemia (AML), ALL, chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL), and hairy cell leukemia (HCL).

6.4.7.3 Gene Chip Technology

Infants and children diagnosed with chemotherapy resistant ALL may in fact have a different type of leukemia. Gene chip technology has been used successfully to categorize 95 % of the leukemias as ALL, AML, or mixed lineage leukemia (MLL). A possible therapeutic target, a gene called FLT3 is expressed only in MLL. The signatures provided by RNA profiling might provide enough information to enable not only determination of prognosis but also stratification of patients for personalized therapy.

6.4.7.4 Hairy Cell Leukemia

HCL, a cancer of the bone marrow, is a well-defined clinicopathologic entity whose underlying genetic lesion is still obscure. HCL results in accumulation of abnormal B lymphocytes in the blood. Approximately 2,000 new cases of HCL are diagnosed annually in the USA and Europe. Whole-exome sequencing identified five missense somatic clonal mutations that were confirmed on Sanger sequencing, including a heterozygous mutation in BRAF that results in the BRAF V600E variant protein (Tiacci et al. 2011). Since BRAF V600E is oncogenic in other tumors, further analyses were focused on this genetic lesion. None of the patients with other peripheral B-cell lymphomas or leukemias who were evaluated carried the BRAF V600E variant. It was concluded that BRAF V600E mutation is present in all patients with HCL, and this finding may have implications for the pathogenesis, diagnosis, and targeted therapy of HCL. It also provides an immediate therapeutic indication for the use of available anti-B-raf drugs. Trovagene has licensed this technology for diagnostic applications, and assay may help physicians monitor the effectiveness of treatment and disease relapse.

6.4.7.5 Laboratory Assessment of Leukemia

At diagnosis, patients with acute leukemia have a total of 10^{11} to 10^{12} malignant cells. Without treatment, the clone continues to expand, and death results when there are ~10^{13} malignant cells. The disease is considered to be in complete remission when the clone decreases to 10^{10} and fewer than 5 % of the cells in the bone marrow samples are morphologically identifiable blasts. From that time until overt clinical relapse, the number of leukemia cells in the body remains largely unknown. Cytological staining, for example, cannot detect neoplastic cells accounting for less than 1 % of a cell population. Differences in gene expression are robust enough to classify leukemias correctly as MLL, ALL, or AML.

Genzyme's FLT3 Mutation Analysis detects FLT3 receptor mutations, which are one of the most common genetic abnormalities in AML and have been shown to be an independent predictor of survival. Approximately 30 % of patients with AML have FLT3 mutations.

Rearrangements of the MLL gene located at chromosome band 11q23 are commonly involved in adult and pediatric cases of primary acute leukemias and also found in cases of therapy-related secondary leukemias. Approximately 50 different chromosomal translocations of the human MLL gene are currently known and associated with high-risk acute leukemia. The large number of different MLL translocation partner genes makes a precise diagnosis a demanding task. After their cytogenetic identification, only the most common MLL translocations are investigated by RT-PCR analyses, whereas infrequent or unknown MLL translocations are excluded from further analyses. To overcome this limitation, a universal long-distance inverse PCR approach has been devised that enables the identification of any kind of MLL rearrangement if located within the breakpoint cluster region. This diagnostic tool has been proven successful for analyzing any MLL rearrangement including previously unrecognized partner genes. Furthermore, the determined patient-specific fusion sequences are useful for MRD monitoring of MLL-associated acute leukemias.

For the diagnosis and monitoring of therapy of leukemias, the F-FISH probe was developed by Cancer Genetic Inc.'s proprietary Signal Exchange Approach. With this method, probes are derived from the regions flanking genes which contain the breakpoints. This probe design addresses the technical limitations associated with currently available FISH products and cytogenetic or molecular methods. Significant advantages of the assay format include:

- Two-color signal recognition of normal and rearranged chromosomes.
- Proximal and distal region specificity.
- Metaphase cell analysis is not necessary as increased information is available at the interphase level.
- Identification of variant and masked translocations.
- Detection of all known breakpoints in the bcr and abl region genes because the probes are derived from the regions outside these breakpoints.
- Detects region-specific deletions on der(9) and der(22) chromosomes.
- False-positive and false-negative signals completely eliminated because of the distinct signal pattern of normal and tumor cells.
- First report on insertion of the BCR–ABL fusion at the ABL–BCR region.

6.4.7.6 Molecular Probes

Molecular probes are used to diagnose acute or chronic leukemia. Both DNA mapping by Southern blotting and PCR can be used to detect chromosomal translocations (e.g., the Philadelphia chromosome) and determine the type of rearrangement. BCR–ABL translocation can be detected and quantified with the use of mRNA down to a level of 10^{-6} (i.e., 1 leukemia cell per 10^6 total cells). Light microscopy, cytogenetic analysis, flow cytometry, in situ hybridization, and Southern blot analysis will not detect malignant cells until their population exceeds 1–5 % of the normal cell total. By contrast, PCR is sufficiently sensitive to detect one leukemia cell among 100,000 or even one million normal cells.

FISH probes directed against the BCR and ABL genes can reliably detect the fusion gene with a sensitivity of 0.05 %. RT-PCR is capable of detecting very low levels of BCR–ABL mRNA transcripts, allowing detection of a single leukemia cell. Real-time PCR can quantify changes in transcript number and thus levels of MRD and is useful in guiding clinical decision-making. This is particularly useful in detecting relapse following allogeneic stem cell transplantation (SCT) and in predicting the likelihood of a durable complete cytogenetic response to interferon. Patients who become 100 % Ph negative on interferon, as detected by routine cytogenetic evaluation of 20 cells (20 % or less of interferon-treated patients), have a wide range of levels of PCR positivity. Only those with low levels of BCR–ABL transcripts will remain cytogenetically negative for prolonged periods.

6.4.7.7 Minimal Residual Disease

Various studies have confirmed that locating MRD in hematological malignancies is a valuable step in halting return of the disease. Molecular genetic and cytoimmunological markers have been applied for the detection of MRD in hematological malignancies. Two molecular diagnostic techniques that have been employed to confirm the presence of MRD in hematological malignancies are:

1. FISH techniques have been used to detect numerous chromosomal abnormalities in interphase and metaphase cells in patients with hematological malignancies. However, the sensitivity of MRD analysis by FISH approaches only 1 %.
2. Various chromosomal alterations in leukemia represent candidates for PCR amplification. RT-PCR assay has proved a useful method not only for detecting MRD but also for quantitatively assessing the residual tumor burden in leukemia. Genzyme's exclusive WT1 RQ-PCR test is designed to detect MRD or very low levels of disease. The WT1 gene is expressed in approximately 90 % of patients with AML.

MRD testing is appropriate in CML following SCT to enable early detection of relapse and following IFN-α therapy to gauge the response. A single qualitative positive RT-PCR test is not predictive of relapse in an individual patient. However, patients who remain RT-PCR positive 6 months post-SCT are at high risk of relapse. Quantitative RT-PCR is of no value in patients being treated with IFN-α. With so many variables, the kinetics of change of the size of BCR–ABL clone is probably the major determinant of outcome of therapy in CML in the context of both SCT and IFN-α.

6.4.7.8 Screening of Gene Mutations in Chronic Myeloproliferative Diseases

The 2008 edition of the WHO document on the classification of CMPDs has incorporated new information on the molecular pathogenesis of BCR–ABL-negative myeloproliferative disorders (MPDs) including the screening for JAK2V617F mutation

(Tefferi and Vardiman 2008). It lists CMPDs as a subdivision of myeloid neoplasms that includes the four classic MPDs: CML, polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), as well as chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia/hypereosinophilic syndrome (CEL/HES), and "CMPD, unclassifiable." The term "CMPDs" is now replaced by "myeloproliferative neoplasms (MPNs)," and the MPN category now includes mast cell disease (MCD), in addition to the other subcategories mentioned above. At the same time, however, myeloid neoplasms with molecularly characterized clonal eosinophilia, previously classified under CEL/HES, are now removed from the MPN section and assembled into a new category of their own. The WHO diagnostic criteria for both the classic BCR–ABL-negative MPDs (i.e., PV, ET, and PMF) and CEL/HES have also been revised by incorporating new information on their molecular pathogenesis. The current review highlights these changes and also provides diagnostic algorithms that are tailored to routine clinical practice.

These revisions were prompted by the discovery of the JAK2V617F mutation, and soon after this discovery, QIAGEN Marseille developed and commercially launched a range of JAK2 mutation assays (MutaScreen™, MutaQuant™) available worldwide to the scientific community. Clinical studies to support the clinical regulatory status of these products are ongoing. In patients suspected of suffering from PV, the association of a JAK2 mutation with nearly all the patients with the disease makes the use of red cell mass measurement to differentiate PV from "secondary" or "apparent" polycythemia useless. Peripheral blood JAK2V617F screening is thus the preferred first test for evaluating a patient with suspected PV. Because JAK2V617F also occurs in ~50 % of patients with either ET or PMF, the WHO panel proposes to include mutation screening in the diagnostic work-up of both diseases. An international collaborative study coordinated by the European Leukemia Network (ELN) consortium on standardization of WT1 testing for risk stratification in AML has been published (Cilloni et al. 2009). The objective of this study was to select the best-performing WT1 assay and to assess the value of WT1 monitoring during AML treatment to estimate the risk of relapse. Results of the study show that the high-performance WT1 assay designed by the ELN group is adapted to MRD assessment. This specific assay developed and validated in the context of this study, WT1 ProfileQuant® (QIAGEN Marseille), is CE marked and can be used with most RQ-PCR instruments. Application of a standardized WT1 assay provides independent prognostic information in AML, lending support to incorporation of early assessment of MRD to develop more robust risk scores, to enhance risk stratification, and to identify patients who may benefit from allogeneic transplantation.

6.4.8 Lung Cancer

Lung cancer is the leading cause of cancer-related death in Western nations and is broadly divided into two types. Small-cell lung cancer (SCLC) accounts for ~80 %

of the all lung cancers and has a potential for cure by surgical resection. NSCLC, an epithelial tumor, comprises about 20 % of all lung cancers but has a highly aggressive clinical course with tendency for early widespread metastases. Molecular diagnosis of lung cancer has an important role in personalizing therapy.

Consensus statements regard that sputum cytology remains the gold standard for assessing the presence of malignant cells. It is recommended that molecular biomarkers should be validated before they are used in early clinical trials, which should consider ethical issues as well. Detection of CRCs in NSCLC has implications for prognosis and chemotherapy. Techniques are available for enriching CTCs from the peripheral blood of NSCLC patients and determining expression levels for cancer-associated genes. CTCs can be detected in NSCLC patients by high-throughput molecular techniques. Further studies are needed to determine the clinical relevance of gene overexpression.

The most common genetic changes associated with lung cancer involve abnormalities of the genes that regulate the cell cycle. Molecular networking of p53 and p16 tumor suppressor genes and K-ras oncogene exerts a crucial impact on cell cycle regulation and appears to be of major clinical significance for lung cancer evaluation. p53, p16, and K-ras evaluations have been used in lung cancer with particular focus on biological and clinical implications, as well as on new molecular approaches to the study of these genes. Sixteen genes that correlated with survival among patients with NSCLC were identified by analyzing microarray data, and risk scores (DUSP6, MMD, STAT1, ERBB3, and LCK) were selected for RT-PCR and decision-tree analysis (Chen et al. 2007). The five-gene signature is closely associated with relapse-free and overall survival among patients with NSCLC.

Immune response manifested by annexins I and II autoantibodies occurs commonly in lung cancer and is associated with high circulating levels of IL-6—an inflammatory cytokine. A proteomic approach using 2D PAGE followed by Western blot analysis in which individual sera were tested for primary antibodies led to the discovery of antiannexins I and/or II in sera from patients with lung cancer. Various biomarkers have been detected in 90 % of cases of lung adenocarcinoma (LAD). The CARET (Carotene and Retinol Efficacy Trial) feasibility study showed that antiannexin antibodies could be detected in serum samples collected a year prior to clinical diagnosis of lung cancer.

In patients with inoperable SCLC, the efficacy of chemotherapy can be predicted early in the course of therapy by baseline values of serum nucleosomes as independent parameters. Prediction of efficacy of chemotherapy in NSCLC takes into consideration staging, age, baseline value of 1 CYFRA 21-1, and area under the curve (AUC) of the values of nucleosomes on days 1–8. In advanced-stage NSCLC, the initial level of serum CYFRA appears to provide more prognostic information than clinical stage. Furthermore, a drop of >27 % in CYFRA after one cycle of therapy adds prognostic information, so that this threshold appears to be an early measure of response to chemotherapy.

Methylation is one of the genetic mechanisms that can alter the characteristics of a normal cell and change it into a cancer cell. Methylation profiling can identify molecular biomarkers in early lung cancer. A fluorescent protein microarray has

been reported to identify and measure multiple NSCLC-associated antibodies and showed how simultaneous measurements can be combined into a single diagnostic assay (Zhong et al. 2005). Measurements of the five most predictive phage proteins were combined in a logistic regression model that achieved 90 % sensitivity and 95 % specificity in prediction of patient samples, whereas leave-one-out statistical analysis achieved 88.9 % diagnostic accuracy among all 81 samples. These data indicate that antibody profiling is a promising approach that could achieve high diagnostic accuracy for NSCLC. These biomarkers were licensed by 20/20 GeneSystems for development into a test for lung cancer.

A lung metagene model based on gene expression profiles can predict the risk of recurrence in patients with early-stage NSCLC (Potti et al. 2006). A sample is taken of the tumor removed during surgery; mRNA is extracted from it and labeled with fluorescent tags before it is placed on a gene chip where it binds to its complementary DNA sequence. When scanned with special light, the fluorescent RNA emits a luminescence that demonstrates how much RNA is present on the chip and thus reveals genes that are most active in a given tumor. A rigorous statistical analysis is used to assess the relative risk of large grouping of genes, called metagenes, which have similar characteristics. The test generates a risk "number" for each patient. If the risk exceeds 50 %, the patient is advised to get chemotherapy. The model predicts recurrence for individual patients significantly better than clinical prognostic factors and is consistent across all early stages of NSCLC. It identified a subgroup of patients who were at high risk for recurrence and who might be best treated by adjuvant chemotherapy. The lung metagene model thus provides a potential mechanism to refine the estimation of a patient's risk of disease recurrence and, in principle, to alter decisions regarding the use of adjuvant chemotherapy in early-stage NSCLC. It is the first genomic test to predict which patients with early-stage NSCLC will need chemotherapy to live and which patients can avoid the toxic regimen of drugs. This is an example of personalized management of lung cancer.

A subset of 11 genes has been identified as a prognostic gene expression signature and validated in multiple independent NSCLC microarray datasets (Navab et al. 2011). Functional annotation using protein–protein interaction analyses of these and published cancer gene expression changes revealed prominent involvement of the focal adhesion and MAPK signaling pathways. Fourteen of the 46 genes were also differentially expressed in LCM primary tumor stroma compared with the matched normal lung. Six of these 14 genes could be induced by TGF-β1 in normal fibroblasts. These results establish the prognostic impact of changes in gene expression of carcinoma-associated fibroblasts in NSCLC patients.

There is an enormous unmet medical need for the diagnosis of lung cancer in the earliest stages when it is most treatable. Serum biomarkers that could aid clinicians in making case management decisions about lung cancer would be extremely useful. Two proteomic platforms were used, and literature was searched to select candidate serum biomarkers for the diagnosis of lung cancer (Patz et al. 2007). Classification and Regression Tree (CART) analysis selected a panel of four serum protein biomarkers—CEA, retinol-binding protein, α1-antitrypsin, and squamous cell carcinoma antigen—that most efficiently predicted which patients had lung cancer.

They were collectively found to correctly classify the majority of lung cancer and control patients in the training set (sensitivity 89.3 % and specificity 84.7 %). These markers also accurately classified patients in the independent validation set (sensitivity 77.8 % and specificity 75.4 %). Remarkably, 90 % of patients who fell into any one of three groupings in the CART analysis had lung cancer. This panel of four serum proteins is valuable in suggesting the diagnosis of lung cancer. The data may be useful for treating patients with an indeterminate pulmonary lesion and potentially in predicting individuals at high risk for lung cancer. Laboratory Corporation of America has a serum protein assay, which could serve as a useful complement to imaging studies such as CT scan to differentiate cancers from benign nodules.

Pretreatment serum MALDI-TOF MS may predict clinical outcome of NSCLC patients treated with EGFR tyrosine kinase inhibitors (TKIs). A clinical study analyzed the association between classification of NSCLC patients by VeriStrat test (Biodesix Inc.) and PFS as well as overall survival and types of clinical progression (Lazzari et al. 2012). VeriStrat "good" classification at baseline based on plasma samples from NSCLC patients treated with gefitinib correlated with longer PFS. Approximately one-third of baseline "good" classifications had changed to "poor" at the time of treatment withdrawal; progression in these patients was associated with the development of new lesions. These findings support the role of VeriStrat in treatment selection of NSCLC patients for EGFR TKI therapy and its potential utility in treatment monitoring.

6.4.8.1 Molecular Subtyping of Lung Cancer

LAD has extreme genetic variation among patients, which is not well understood and limits progress in research and development of therapy. LAD molecular subtypes are a validated stratification of naturally occurring gene expression patterns and encompass different functional pathways and patient outcomes. Different subtypes may be the result of mutations and alterations in gene expression. LAD molecular subtypes (Bronchioid, Magnoid, and Squamoid) were tested for association with gene mutations and CNVs using statistical methods and published cohorts (Wilkerson et al. 2012). A novel validation cohort was assayed and interrogated to confirm subtype–alteration associations. Mutation rates of genes (EGFR, K-ras, STK11, TP53), chromosomal instability, regional copy number, and genome-wide DNA methylation were significantly different among tumors of the molecular subtypes. Secondary analyses compared subtypes by integrated alterations and patient outcomes. Tumors having integrated alterations in the same gene associated with the subtypes, e.g., mutation, deletion, and underexpression of STK11 with Magnoid and mutation, amplification, and overexpression of EGFR with Bronchioid. The subtypes also associated with tumors having concurrent mutant genes, such as K-ras–STK11 with Magnoid. Overall survival of patients, cisplatin plus vinorelbine therapy response, and predicted gefitinib sensitivity were significantly different among the subtypes. The study concluded that LAD intrinsic molecular subtypes co-occur with grossly distinct genomic alterations that affect response to therapy. These results advance

the understanding of etiology of LAD and help in selection of patient subgroups for future evaluation of treatment response. Lung Subtype Platform (LSP™) is being developed commercially by GeneCentric.

6.4.9 Melanoma

Although both benign and malignant lesions result from sun exposure, 10 % of all patients demonstrate an inherited disposition to develop dysplastic nevi and melanoma. Familial dysplastic nevus syndrome is associated with a high rate of melanoma but has not been ascribed to a specific molecular defect. In other families, melanoma is associated with mutations of one of two genes: p/16CDKN2 and CDK4. These genes are components of the retinoblastoma pathway that controls cell proliferation. Such defects account for less than 50 % of cases of familial melanoma.

Molecular mechanisms of melanoma progression are being studied by DNA microarray technology that can enable the elucidation of the exact mechanisms and defects in genetic aberrations. Further advances will enable the identification of global gene expression alterations that are involved in the development of malignant melanoma. These lines of investigation hold promise in improving screening methods to identify those individuals at increased risk of developing melanoma and in developing of treatments using "gene-directed" therapy. MELARIS® (Myriad Genetics) is a predictive medicine test for inherited susceptibility to melanoma and pancreatic cancer. It detects inherited mutations in the p16 gene (CDKN2A or INK4A), which occur in up to 40 % of families with hereditary melanoma. Early detection of lesions is critical for successful treatment.

6.4.10 Ovarian Cancer

On average, US women have a 2 % risk of developing ovarian cancer by age 70. Risk factors for ovarian cancer include previous breast cancer, family history of breast or ovarian cancer, and HNPCC. Thus, ~10 million women in the USA are at risk for the development of ovarian cancer and should be tested for it. Approximately 22,000 new cases are diagnosed annually in the USA out of 200,000 new cases a year worldwide. Most women are diagnosed with ovarian cancer in late-stage disease and have a 5-year survival rate of <30 %, but these rates soar to >90 % if the disease is discovered when cancer still is localized to the ovaries.

Clinical diagnosis of ovarian tumors is difficult in the absence of physical symptoms as the ovaries are located deep in the abdomen. Unfortunately, current methods of diagnosis, such as transvaginal ultrasound, laparoscopy, or PET scan are impractical for general testing as they are complex procedures and would pose a tremendous burden on the healthcare system. The need for an accurate yet simple test has

prompted investigators to explore novel, rapid ways of detecting ovarian cancer. To be practical for testing the high-risk population, a rapid detection assay for ovarian cancer should have the possibility to be performed on an easily obtained specimen, such as a blood sample. An assay that requires a tissue biopsy, for example, would be ruled out as a convenient test. The assay must be robust, such that normal handling and transport of the specimen to the testing laboratory does not alter the analyte or biomarker being measured. The need for special sample processing not normally performed at the patient service center such as flash freezing or extraction of a particular molecular component could make it difficult to adapt the test for routine use. In the laboratory, the process for testing the sample should be high throughput and automated. Several tests are in development for ovarian cancer. Some of these are discussed in the following sections.

6.4.10.1 Mutation of Genes

Sporadic ovarian tumors are the end result of a complex pathway involving multiple oncogenes and tumor suppressor genes, including HER-2/neu, K-ras, p53, BRCA1, and additional tumor suppressor genes on chromosome 17. Recent studies indicate that germline mutations of the BRCA1 gene confer a lifetime risk of approximately 45 % for ovarian cancer in families with multiple cases of such cancer (this gene is also involved in breast cancer). In the general population, mutations in the BRCA1 gene occur in approximately 5 % of women in whom ovarian cancer is diagnosed before the age of 70 years. Protein truncation test outperforms single-strand conformation polymorphism (SSCP) analysis for BRCA1 mutation detection in ovarian cancer.

Mutations at the p53 tumor suppressor gene locus are frequently associated with human ovarian carcinoma. A multiplex PCR screening assay has been used to amplify the complete p53 coding region from genomic DNA in a single step. Deletions and insertions were subsequently found in the newly established ovarian carcinoma cell lines.

6.4.10.2 Relevance of Genetic Testing to Management of Ovarian Cancer

In families at high risk of hereditary breast or ovarian cancer, detection of a mutation in BRCA1 or BRCA2 is required in order to know which individuals in the family have inherited mutations and should consider preventive strategies. Removal of the ovaries from women with a mutation in either the BRCA1 or BRCA2 gene who have completed childbearing is completely effective in reducing the risk of cancer of the ovaries. Additionally, the procedure reduced cancer of ovary-related tissue remaining after ovary removal by >90 % and simultaneously reduces the risk of breast cancer by >50 %. The effectiveness of prophylactic oophorectomy in carriers of BRCA mutations provides a strong rationale for genetic testing in women with a strong family history of breast cancer.

6.4.10.3 Biomarkers of Ovarian Cancer

Diagnosis of ovarian cancer prior to clinical manifestations can be established by study of serum biomarkers. Several biomarkers have been reported to be useful for the detection of ovarian cancer.

Lysophosphatidic acids (LPAs), a category of phospholipids, were suspected to be related to ovarian cancer because of their role in stimulating cell proliferation. The combined plasma concentrations of two LPA categories, 16:0 LPA and 20:4 LPA, could classify cancer and noncancer samples with 93 % accuracy. The two lipid biomarkers show promise for early detection, as they could classify early-stage (I/II), as well as later-stage disease, with high accuracy.

MS pattern analysis is a potentially rewarding approach in that it utilizes the power of combined multiple biomarkers so that, in principle, discrimination accuracy is higher. Having reliable, discriminatory patterns obviates the need to identify and purify the markers of interest and develop molecular assays for them. This process can be quite tedious, especially if the biomarkers are in low concentration. Furthermore, MS pattern assays take advantage of the high resolving power and small sample volume requirement of MS. MS pattern analysis requires laboratories to develop new ways to continually affirm platform and sample integrity in the absence of biology-based means. Assay variability could arise from potential heterogeneity of molecules within a spot, which is why SELDI-TOF employs multiple desorptions from different positions within a spot.

Various studies have shown that a 3-biomarker panel could classify stage I/II ovarian cancer samples from healthy control samples with 97 % specificity and 74 % sensitivity, compared to 97 % specificity and 54 % sensitivity when CA-125 alone was used to classify. Even though considerably better than the use of CA-125, it does not meet the requirements for a screening test. The 3-biomarker panel would have greater value if used in conjunction with another complementary test. CA-125 and one recently approved by FDA called HE4 are used to track whether chemotherapy is working or cancer is recurring. A one-time CA-125 test cannot screen seemingly healthy women because levels rise with benign cysts, endometriosis, and even normal menstruation, but Fujirebio's triage test uses HE4 and CA-125 to assess who most likely has a benign cyst and who has cancer.

The FDA-approved OVA1 test (Vermillion) is an IVD multivariate index test that combines the results of five immunoassays using a proprietary unique algorithm to produce a single numerical score indicating a women's likelihood of malignancy. OVA1 test is used by physicians as an adjunctive test to complement, not replace, other diagnostic and clinical procedures.

OvaSure, which measures concentrations of leptin, prolactin, osteopontin, insulin-like growth factor II, macrophage inhibitory factor, and CA-125 by using a multiplex, bead-based immunoassay system, is a screening test for women at high risk of ovarian cancer that was developed by Yale University under a law that allows a single laboratory to offer testing without FDA review. Used on blood samples stored from cancer patients and healthy women, the test correctly identified

cancer a sensitivity of 95.3 % and a specificity of 99.4 % (Visintin et al. 2008). However, this does not prove that OvaSure can detect when cancer is forming. This test was later found to be useless.

6.4.10.4 Concluding Remarks on Testing for Ovarian Cancer

The multiple ovarian cancer detection tests under development are based upon different, complementary technologies and disparate biomarkers, so in principle their combined use will provide higher accuracy. Suboptimal sensitivity of a detection assay can be compensated somewhat through regular testing of women at high risk; a convenient assay makes such routine testing less burdensome and increases patient compliance. These arguments suggest that an assay with even 95 % sensitivity and specificity should help in the management of ovarian cancer.

A major advantage of isolating discrete biomarkers is that immunoassays or other biomolecule-specific assays can be developed for their detection. Immunoassays are performed routinely in the clinical lab on automated platforms with high throughput and, as such, are more economical and practical than SELDI-TOF in its present form. However, development of sufficiently sensitive immunoassays would be required.

6.4.11 Pancreatic Cancer

The survival rate of pancreatic cancer patients is the lowest among those with common solid tumors, and early detection is one of the most feasible means of improving outcomes. In conventional practice, the use of CA 19-9 levels and imaging techniques is not optimal for detecting small pancreatic lesions.

6.4.11.1 Proteomic Techniques for Diagnosis of Pancreatic Cancer

Quantitative proteomics has shown great potential for the study of cancer and has opened new opportunities to investigate crucial events underlying pancreatic tumorigenesis and to exploit this knowledge for early detection and better intervention. Isotope-coded affinity tag technology for proteomic analysis of human cancer tissue has been used to identify differentially expressed proteins in pancreatic cancer. Proteomic analysis of pancreatic juice reveals hepatocarcinoma–intestine–pancreas/pancreatitis-associated protein (HIP/PAP) and protein that is 85 % identical to HIP/PAP, which has been designated as PAP-2. These proteins identified can be directly assessed for their potential as biomarkers for pancreatic cancer by quantitative proteomic methods or immunoassays. One study has compared plasma proteomes between pancreatic cancer patients and sex- and age-matched healthy controls using SELDI coupled with hybrid quadrupole TOF MS (Honda et al. 2005). A discriminating proteomic pattern was extracted from the data using a

support vector machine learning algorithm and was applied to two validation cohorts. A set of four mass peaks most accurately discriminates cancer patients from healthy controls with sensitivity of 97.2 % and specificity of 94.4 %. When combined with CA 19-9, 100 % of pancreatic cancers, including early-stage tumors, were detected. Although a multi-institutional large-scale study will be necessary to confirm clinical significance, the biomarker set identified in this study may be applicable to using plasma samples to diagnose pancreatic cancer.

6.4.11.2 Detection of K-ras Mutations in Pancreatic Cancer

Trovagene recently completed the analytical development of digital PCR assays for detecting the most common K-ras mutations, including ones accounting for 95 % of K-ras mutations found in pancreatic adenocarcinomas. In collaboration with the MD Anderson Cancer Center, Trovagene is developing a test for the detection of transrenal K-ras mutations in the urine of patients with pancreatic cancer, which could eventually lead to a sensitive method for staging tumors before treatment and detecting MRD after treatment.

6.4.12 Prostate Cancer

Prostate cancer is the most common cause of death from cancer in men over the age of 75. Over 230,000 new prostate cancer cases and 30,000 prostate cancer deaths are estimated in the USA per year. This makes prostate cancer the most commonly diagnosed cancer and the second leading cause of cancer-related deaths in men in the USA. At present, measurement of serum PSA is the most useful biomarker for early detection, clinical staging, and monitoring. However, although on average men with prostate cancer have higher levels of PSA than healthy men or those with benign prostate diseases, there is a wide variation in levels throughout the population, which leads to false positives and unnecessary biopsies. Although the optimal use of PSA testing remains controversial, studies indicate that PSA screening does reduce mortality. Significant and widespread differences in gene expression patterns exist between benign and malignant growth of the prostate gland. Gene expression analysis of prostate tissues should help to disclose the molecular mechanisms underlying prostate malignant growth and identify molecular markers for diagnostic, prognostic, and therapeutic use.

6.4.12.1 Early Detection of Prostate Cancer Recurrence by Nanotechnology

An automated gold nanoparticle bio-barcode assay probe has been described for the detection of PSA at 330 fg/mL, along with the results of a clinical pilot study

designed to assess the ability of the assay to detect PSA in the serum of 18 men who have undergone radical prostatectomy for prostate cancer (Thaxton et al. 2009). Available PSA immunoassays are often not capable of detecting PSA in the serum of men after radical prostatectomy. This new bio-barcode PSA assay is approximately 300 times more sensitive than commercial immunoassays, and all patients in this study had a measurable serum PSA level after radical prostatectomy. Because the patient outcome depends on the level of PSA, this ultrasensitive assay enables (1) informing patients, who have undetectable PSA levels with conventional assays but detectable and nonrising levels with the barcode assay, that their cancer will not recur; (2) earlier detection of recurrence because of the ability to measure increasing levels of PSA before conventional tools can make such assignments; and (3) use of PSA levels, which would otherwise not be detectable with conventional assays, to follow the response of patients to treatment.

6.4.12.2 Gene Expression Analysis of Prostate Cancer

Gene expression levels have compared with two reference samples—normal prostate tissue from men with prostate cancer and prostate samples from men with no history of prostate disease. Expression of two proteins, hepsin (the membrane-spanning serine protease) and PIM1 (an oncogenic kinase), is significantly correlated with poor prognosis in prostate cancer. These biomarkers are unlikely to replace PSA in the clinic for routine screening, as taking a blood sample is far less invasive than a biopsy; however, when biopsy material is available, these cell-associated markers might be useful for identifying candidates for prostatectomy.

One novel prostate cancer gene identified by gene expression analysis is hepsin—a serine transmembrane protease that is overexpressed in cancer cells from both primary and metastatic tumors. Another gene, AMACR, is upregulated only in localized prostate cancer tumors, but not metastatic. This gene is more reliable than PSA for identifying aggressive prostate tumors; when gene expression patterns of additional prostate cancer patients were evaluated, AMACR expression alone can predict which patients had aggressive prostate tumors, with 97 % specificity and 100 % sensitivity.

Quantitative RT-PCR assays show that a four-gene expression signature for prostate cancer cells (consisting of UAP1, PDLIM5, IMPDH2, and HSPD1) can detect Gleason grade 3 and grade 4 cancer cells in prostate tissue (Guyon et al. 2009). This may be useful as an adjunct test to the pathology examination of prostate tissue taken at biopsy or prostatectomy.

6.4.12.3 Huntingtin Interacting Protein 1

Huntingtin Interacting Protein 1 (HIP1) is an oncogene for prostate, colon, and brain cancers. In addition to human cancers, HIP1 is also overexpressed in prostate tumors from the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model. Autoantibodies to HIP1 developed in the sera of TRAMP mice with prostate

cancer as well as in the sera from human prostate cancer patients have led to the development of an anti-HIP1 serum test in humans that had a similar sensitivity and specificity to the anti-alpha-methylacyl-CoA racemase (AMACR) and PSA tests for prostate cancer and when combined with the anti-AMACR test yielded a specificity of 97 % (Bradley et al. 2005). These data suggest that HIP1 plays a functional role in carcinogenesis and that a positive HIP1 autoantibody test may be an important serum marker of prostate cancer.

6.4.12.4 Integrative Genomic and Proteomic Profiling of Prostate Cancer

Molecular profiling of cancer at the transcript level has become routine. Large-scale analysis of proteomic alterations during cancer progression has been a more daunting task. High-throughput immunoblotting has been used to interrogate tissue extracts derived from prostate cancer. This approach has identified numerous proteins that are altered in prostate cancer relative to benign prostate. Integrative analysis of this compendium of proteomic alterations and transcriptomic data reveals only 50–60 % concordance between protein and transcript levels. However, differential proteomic alterations between metastatic and clinically localized prostate cancer that maps concordantly to gene transcripts serve as predictors of clinical outcome in prostate cancer as well as other solid tumors.

6.4.12.5 LCM for Diagnosis of Prostate Cancer

The proportion of unbound serum PSA (percent-free prostate-specific antigen [fPSA]) is reported to be lower in men with prostate cancer compared to men with benign prostates. The majority of immunoreactive PSA in serum is complexed to alpha-1-antichymotrypsin (ACT). LCM has been used to assess the bound versus free form of intracellular PSA in both benign and malignant epithelium procured from prostate tissue. Western blotting analysis of 1D PAGE gels reveals that in the vast majority of intracellular tumor, normal PSA exists within cells in the "free" form. Binding studies show that PSA recovered from LCM-procured cells retained the full ability to bind ACT, and 2D PAGE Western analysis demonstrates that the PSA/ACT complex is stable under strong reducing conditions. Intracellular PSA, therefore, exists in the "free" form and that binding to ACT occurs exclusively outside of the cell.

PSA or histological examination of bulk tissue may not accurately reflect molecular events that take place in the actual ductal epithelium that change as a consequence of the malignant process in the prostate gland. Alternative proteomic-based approaches including LCM enable the identification of protein markers in the actual premalignant and frankly malignant epithelium. The phenotype of a given cell type is ultimately determined by the composition and activation status of its proteins. Therefore, quantitative and qualitative proteomic measurement of normal and neoplastic prostate cells is an important experimental approach that will complement genomic DNA and gene expression analyses. The NCI Prostate Group has

been studying protein profiles of prostate cancer using tissue microdissection and two protein analysis methods: 2D PAGE and SELDI.

SELDI ProteinChip MS technology has been used for the rapid, reproducible, and simultaneous identification of four well-characterized prostate cancer-associated biomarkers: PSA (free and complexed forms), prostate-specific peptide, prostate acid phosphatase, and prostate-specific membrane antigen in cell lysates, serum, and seminal plasma. Proteins corresponding to the mass of these biomarkers can readily be captured and detected using either chemically defined or antibody-coated ProteinChip arrays. Several other proteins are upregulated in cell lysates of pure populations of prostate cancer-associated cells procured by LCM when compared with mass spectra of normal cell lysates. Coupling LCM with SELDI provides tremendous opportunities to discover and identify the signature proteins associated with each stage of tumor development.

6.4.12.6 PCA3 Gene Detection in Urine

A urine test for prostate cancer based on detection of prostate cancer 3 gene (PROGENSA's PCA3 assay) was cleared by the FDA in 2012. This is the first gene-based, adjunctive screen for this prostate cancer, which helps in determining whether men who have had previous negative biopsies need repeat biopsies. PCA3 has an mRNA product that has been shown to be overexpressed only in malignant prostate tissue. Quantitative RT-PCR assay for PCA3 shows a 66-fold upregulation of PCA3 in patients with prostate cancer. This assay is a valuable tool for the detection of malignant cells in blood, urine, or other clinical specimens, and it has important implications for the earlier diagnosis and molecular staging of prostate cancer. A study has shown that PCA3 is independent of prostate volume, serum PSA level, and the number of prior biopsies (Deras et al. 2008). The quantitative PCA3 score correlated with the probability of positive biopsy, and logistic regression results suggest that the PCA3 score could be incorporated into a nomogram for improved prediction of biopsy outcome. The results of this study provide further evidence that PCA3 is a useful adjunct to current methods for prostate cancer diagnosis. The test offers advantages over PSA testing, the current standard for initial prostate cancer screening in conjunction with a digital rectal exam. It has great potential in reducing the number of unnecessary biopsies. The PCA3 test is now available through laboratories in the USA using PCA3 analyte-specific reagents from Gen-Probe and in Europe as the CE-marked PROGENSA™ PCA3 in vitro assay.

A multiplex panel of urine transcripts was shown to outperform PCA3 transcript alone for the detection of prostate cancer (Laxman et al. 2008). The test is based on the finding that gene fusions are common in prostate cancer, and that by overriding molecular switches that turn off excess growth, they may be the causative factor in some forms of the disease. The investigators considered six gene fusions in addition to PCA3 as putative prostate cancer biomarkers: TMPRSS2, which fuses with either ERG or ETV1 (two genes known to be involved in several types of cancer), and another five genes that fuse on to ERG or ETV1 to cause prostate cancer. They measured the expression of these seven biomarkers in sedimented urine using qPCR on patients presenting for

biopsy or radical prostatectomy. By univariate analysis, they found that increased GOLPH2, SPINK1, and PCA3 transcript expression and TMPRSS2–ERG fusion status were significant predictors of prostate cancer. Multivariate regression analysis showed that a multiplexed model, including these biomarkers, outperformed serum PSA or PCA3 alone in detecting prostate cancer. The urine test that screens for the presence of four different RNA molecules accurately identified over 75 % of patients (5 % better than with PCA3 alone), who were later found to have prostate cancer, and was 61 % effective in ruling out disease in other study participants. These results provide the basis for the development of highly optimized, multiplex urine biomarker tests for more accurate detection of prostate cancer.

6.4.12.7 Prolaris Test

Prolaris (Myriad) test, a 46-gene expression assay, is used to predict men who are at a heightened risk of biochemical recurrence of prostate cancer and therefore should receive more aggressive therapy. In 2012, Myriad, in collaboration with Intermountain Healthcare (Salt Lake City, UT), started PRO 008, a project to analyze biopsy samples from 200 patients who have been diagnosed with prostate cancer. The goal is to demonstrate the prognostic ability of Prolaris to assess a patient's risk of biochemical recurrence of disease and death resulting from it.

6.4.12.8 Prostate Biopsy for Detection of Prostatic Intraepithelial Neoplasia

High-grade prostatic intraepithelial neoplasia (PIN) has been established as a premalignant lesion of the prostate that has a high potential to progress to invasive prostate cancer. Every year ~1.5 million prostate biopsies are performed in the USA to detect new cases of prostate cancer. High-grade PIN is found in an average of 9 % of prostate biopsies which represents an estimated 135,000 new cases of high-grade PIN diagnosed each year. Patients who are found to have high-grade PIN are at high risk of prostate cancer with up to 37 % of patients being later diagnosed with prostate cancer within 1 year.

PIN may prove to be an important diagnostic indicator of cancer of prostate. The development of assays for the accurate detection of PIN could be a component in the treatment of this disease. A proprietary panel of proPSA serum markers from Beckman Coulter may help diagnose the presence of PIN and the earliest progression to prostate cancer.

6.4.12.9 Prostate Core Mitomic Test™

Prostate Core Mitomic Test™ (Mitotics Inc.) has been developed to identify men at high risk of having a prostate tumor missed by the prostate biopsy procedure, without the need for further invasive biopsy procedures. It is based on the finding

that several cancers are characterized by large-scale mtDNA deletions and one of these deletions has potential utility in resolving false-negative from true-negative prostate needle biopsies. In a study, qPCR assay was used to measure the levels of the deletion in individual negative needle biopsies in patients who had a repeat biopsy within a year with known outcomes. The test was shown to have a sensitivity of 84 % with a negative predictive value of 91 % (Robinson et al. 2010). Advantages of this test are:

- Detects elevated deletion levels in normal-appearing cells that coexist with a tumor
- Uses existing biopsy tissue
- Reduces cost by using a simple, cost-effective, high-throughput laboratory process
- Simplifies testing

6.4.12.10 Prostate Health Index

Beckman Coulter's Prostate Health Index (PHI) blood test is used for detecting prostate cancer in patients with PSA values in the 4–10 ng/mL range. Men with PSA levels in this range are normally considered for prostate biopsy, even though the PSA elevation may have a benign basis. The PHI test is designed to help differentiate prostate cancer from benign conditions and reduce the need for biopsy. PHI essentially combines three automated blood tests into one index that estimates a man's probability of having prostate cancer found on biopsy. The test thus generates a composite score of Access Hybritech PSA, Access Hybritech fPSA, and Access Hybritech p2PSA. The assay is 2.5 times more specific in detecting prostate cancer than PSA in this subset of patients and in clinical trials reduced the number of unnecessary biopsies carried out by 31 %.

6.4.12.11 Screening of Multiple SNPs for Risk of Prostate Cancer

SNPs in five chromosomal regions, three at 8q24 and one each at 17q12 and 17q24.3, have been associated with prostate cancer. Whereas each SNP has only a moderate association, SNPs in five chromosomal regions plus a family history of prostate cancer have a cumulative and significant association with prostate cancer (Zheng et al. 2008). Approximately 90 % of the men in the study had one or more of the gene variants, and more than half had two or more. The cancer risk increased as the number of variants rose and increased substantially when men had four or five of the variants. Men with four or five variants made up only 46 % of the study population but had a 4.5-fold increased risk of having prostate cancer compared with men who had none of the variants. If the men also had a family history of prostate cancer, their risk was nearly ten times higher than that of men with none of those risk factors. Another study showed that genetic variants located in nine regions have a cumulative association with prostate cancer risk (Helfand et al. 2010). Identification of an increasing number of SNPs may provide greater understanding of their combined relationship with risk of prostate cancer and disease aggressiveness.

6.4.12.12 Semen Testing for Prostate Cancer Biomarkers

The proportion of free and complex prostate-specific antigen (cPSA) in serum is used for differentiating between benign and malignant prostate disease. To further understand the physiological relationship between PSA in seminal plasma and blood, fPSA and cPSA have been analyzed in blood and in seminal plasma in young healthy men. fPSA in blood, but not cPSA, is associated to PSA in semen. In blood, cPSA, but not fPSA, increases with age in healthy men, which may reflect an increasing incidence of prostate disease.

A semen-based prostate cancer test, designed to improve on the accuracy of the PSA test, has identified a prostate cancer biomarker associated with human carcinoma antigen (HCA). When present along with HCA, the biomarker will form the basis of a new prostate cancer diagnostic test being developed by Proteome Systems.

Ambrilia Biopharma's PSP94 (prostate secretory protein of 94 amino acids) immunoassay is a simple, potential diagnostic tool to determine the stage, grade, and aggressiveness of prostate cancer. PSP along with PSA and prostate acid phosphatase (PAP) are the three most abundant proteins in seminal fluid. Levels of PSA above 4 ng/mL of blood can signal prostate cancer, but not always. As prostate cancer advances, the serum PSP94 level is seen to initially increase but then drops in highly advanced cancer. Ambrilia's immunoassay measures the amount of free PSP94, bound PSP94, and PSP94-binding protein present in the serum. Studies have also shown that levels of PSP94 are hormone independent and not affected by androgen or antiandrogen therapy. PSP94 assessments are therefore likely to be of use during periods of androgen ablation therapy.

6.4.13 Thyroid Cancer

Approximately 4–7 % of the general population develops a clinically significant thyroid nodule during their lifetime. The incidence of papillary thyroid carcinoma (PTC) is growing faster than any other type of malignancy, and the global estimate for all new cases in 2012 is <500,000 with 56,460 cases in the USA alone out of 450,000 thyroid nodule biopsies. It is also important to distinguish between benign and malignant tumors of thyroid. Although FNA cytology is useful in the diagnosis of PTC, the results are inconclusive in many cases. The accuracy and utility of FNA biopsy would be greatly facilitated by the development of specific biomarkers for PTC and its common variants. Numerous potential biomarkers of thyroid cancer have been reported in expression profiling studies, and it is difficult to evaluate them.

6.4.13.1 Afirma Gene Expression Classifier for Inconclusive Thyroid Biopsies

The CLIA-certified Afirma Gene Expression Classifier (Veracyte) measures the expression of 142 genes to reclassify ambiguous results of FNA of thyroid nodules

as either benign or possibly cancerous and is offered as part of Afirma Thyroid FNA Analysis, which combines an initial review based on cytopathology assessment of thyroid nodule FNAs with the Afirma test to resolve inconclusive results. The test is available through a global co-promotion partnership with Genzyme, a Sanofi company. In 2012, the New York State Department of Health cleared the Afirma molecular diagnostic test for inconclusive thyroid nodule results, and Memorial Sloan–Kettering Cancer Center will become one of the first institutions in the state to offer the test.

6.4.13.2 Gene Expression in Thyroid Cancer

Quest Diagnostics' Thyroid Cancer Mutation Panel aids in detecting cancer in thyroid biopsies, which are found to be indeterminate for cancer by current cytology test methods. Approximately 15–20 % of these biopsies, which are collected by FNA, produce indeterminate results. About 300,000 thyroid FNA biopsy procedures are performed annually in the USA. The new panel identifies mutations of the molecular biomarkers BRAF V600E, RAS, RET/PTC, and PAX8/PPAR gamma, which are associated with papillary and follicular thyroid cancer, two common forms of the disease.

6.4.13.3 Multiple Endocrine Neoplasia Type 2B as Risk Factor for Thyroid Cancer

Multiple endocrine neoplasia type 2B (MEN2B) is an autosomal-dominant inherited cancer syndrome. MEN2B patients have a high risk of developing medullary thyroid carcinoma (MTC), and prophylactic thyroidectomy is recommended by 6 months of age. Genetic testing can identify MEN2B patients before cancer progression. Two RET proto-oncogene mutations, in exon 15 at codon 883 and in exon 16 at codon 918, account for more than 98 % of MEN2B cases. An assay using unlabeled probes and the LightCycler 480 instrument was developed to genotype these two common MEN2B RET mutations (Margraf et al. 2008). This is a rapid, closed-tube method that is less time consuming and less expensive than sequencing. This assay demonstrates 100 % specificity and sensitivity for the identification of RET mutations causative of MEN2B.

6.4.13.4 miRNA Expression Profiling in Thyroid Cancer

miRNA expression profiling correlates with various cancers, with these genes thought to act as both tumor suppressors and oncogenes. ret/PTC1 is an oncogene with constitutive kinase activity implicated in the development of PTC. This rearrangement leads to aberrant MAPK activation that is implicated in PTC tumorigenesis.

A cell line matrix model containing normal thyroid, PTC with native BRAF mutation or ret/PTC activation, and normal thyroid cells transfected with either BRAFmut or ret/PTC has been used to investigate transcription and posttranscriptional regulation in PTC using DNA microarray and miRNA analysis (Cahill et al. 2006b). Ambion's RecoverAll™ Total Nucleic Acid Isolation Kit was used to extract RNA for expression microarray and miRNA analysis. The Applied Biosystems TaqMan® Human Early Release Panel containing 160 microRNA assays designed to detect and quantify mature miRNAs was used to analyze the cell line panel. Distinct gene signatures were found contingent upon the primary genetic mutation (ret/PTC or BRAFmut). A unique miRNA expression signature differentiated between PTC cell lines with ret/PTC, BRAFmut, and a normal thyroid cell line. As miRNAs are stable, abundant, and very easily detectable, they may be ideal candidates for diagnostic biomarkers. They are also resilient and detectable in archival material. Future exploration on a larger cohort of samples will hopefully consolidate the correlation between these miRNAs and their host genes in PTC.

6.4.14 Urinary Bladder Cancer

Tumors arising from the urothelial mucosa lining the urinary bladder are the most common malignancy of bladder and upper urinary tract (ureters and renal pelvis). A multitarget, multicolor FISH assay has been developed for the detection of urothelial carcinoma (UC) in urine specimens. A FISH assay containing centromeric probes to chromosomes 3, 7, and 17 and a locus-specific probe to band 9p21 has high sensitivity and specificity for the detection of bladder cancer from voided urine specimens. A multicenter, blinded trial has shown that multitarget UroVysion™ (Abbott) FISH assay is significantly more sensitive than voided cytology for detecting bladder cancer in patients with hematuria and no history of bladder cancer (Sarosdy et al. 2006). Based on these data, UroVysion™ was approved by the FDA for use in patients with hematuria.

UroCor Inc. (now part of Dianon Systems Inc., a subsidiary of Laboratory Corporation of America) has developed a test for direct identification of p53 gene mutations in patients with bladder cancer utilizing a urine specimen.

NMP22 BladderChek (Matritech Inc.) is a POC test for bladder cancer that returns results while the patient is in the physician's office. Current tests performed in a central laboratory take 2–3 days to deliver results. NMP22 BladderChek measures the level of NMP22, a nuclear matrix protein. The test would be used in conjunction with cystoscopy, a procedure in which a fiber-optic tube is inserted into the bladder through the urethra permitting visual examination of the bladder.

ImmunoCyt™/uCyt+™ (DiagnoCure), a noninvasive test for detecting superficial bladder cancer through a simple urine sample, detects the presence of cancerous cells with the help of a fluorescence microscope. It has FDA clearance for commercialization in the USA for the monitoring of bladder cancer.

6.5 Role of Molecular Diagnostics in the Management of Cancer

Management of cancer is a multidisciplinary effort in which molecular diagnostics play a key role. The role extends from detection prior to clinical manifestations, confirmation of clinical diagnosis, as a guide to therapy and to follow-up which may span the entire life of a cancer patient.

6.5.1 Risk Assessment and Prevention of Cancer

Detection of mutations is important for risk assessment and prevention of cancer. Tests with the greatest potential for risk assessment include those that target mutations in the following genes:

- BRCA1 and BRCA2 (for breast and ovarian cancers)
- MLH1 and MSH2 (for colon cancer)
- APC (for FAP)
- RET (for medullary thyroid cancer)
- TP53 (for several tumors)
- CDKN2A (for melanoma)
- RB1 (for retinoblastoma)

Detection of mutation in an individual would theoretically lead to increased surveillance. Lifestyle changes might be advised to avoid known risk factors for progression of cancer. In some cases, prophylactic surgery may be recommended. In addition, some chemotherapeutic agents might be prescribed on a preventive basis. Detection of a mutation may be followed by surveillance-oriented examinations, including those involving colonoscopy, mammography, measurement of PSA, and other tests. This tactic will promote the early detection of cancer and early management. Current molecular research is expected to reveal other markers for early diagnosis of cancer. In addition, the possibility of generating genetic profiles for individual tumors offers unique opportunities for distinguishing between metastases and primary tumors.

6.5.2 Role of Molecular Diagnosis in the Design of Future Cancer Therapies

A better understanding of cancer biology would enhance the design of future therapies for cancer. For example, PCR can already be used to assess the efficacy of new therapies for leukemias. Various biomarkers of cancer can be used for diagnosis as well as design of therapies. Furthermore, they can be used for testing the efficacy and toxicity of drug candidates.

Future targets for cancer therapies may include defective proto-oncogenes or the tumor suppressor genes themselves. A gene therapy strategy might be employed to

correct or replace the defective gene. In cancers with multifactorial etiology, it may be possible to interrupt one or two steps in the complex pathways, thereby hindering the overall evolution of the tumor. SAGE studies have demonstrated that tumor and normal endothelium are distinct at the molecular level, a finding that may have significant implications for the development of antiangiogenic therapies.

6.5.3 Molecular Classification of Cancer

The optimal treatment of patients with cancer depends on establishing accurate diagnoses by using a complex combination of clinical and histopathological data. In some instances, this task is difficult or impossible because of atypical clinical presentation or histopathology. Efforts have been made to determine whether the diagnosis of multiple common adult malignancies could be achieved purely by molecular classification. Overall classification accuracy far exceeds the accuracy of random classification. Poorly differentiated cancers cannot be accurately classified according to their tissue of origin, indicating that they are molecularly distinct entities with dramatically different gene expression patterns compared with their well-differentiated counterparts. These results demonstrate the feasibility of accurate, multiclass molecular cancer classification and suggest a strategy for future clinical implementation of molecular cancer diagnostics.

6.5.4 Determination of Cancer Prognosis

Prognosis involves not only predicting the future course of the disease but also the response to treatment. Information obtained from tumor classification techniques can be used to predict tumor behavior. Molecular diagnostics provide an easier, less invasive way to determine cancer prognosis. For example, patients with the greatest degree of amplification (in terms of gene copy numbers) of the N-myc gene in neuroblastoma, a highly malignant tumor, have the worst prognosis. Molecular tests for TP53 and RER are already considered to offer prognostic value in certain types of cancer. In addition, the ability to locate residual cancer by molecular methods can aid in predicting the course of the disease. Gene expression analysis can be applied to determine the prognosis of cancer and may form the basis of individualized treatment for cancer.

6.5.5 Selection of Anticancer Drugs Based on Molecular Diagnosis

Cancer cells have defects within their systems related to the control of the cell cycle. These modifications may, however, confer selective sensitivity to appropriately

designed drug therapy. Thus, molecular defects could potentially be linked to specific drug sensitivities. Such correlations might guide the selection of drugs for therapy based on the molecular diagnosis of individual tumors. An example is the treatment of breast cancer with trastuzumab (Herceptin; Genentech, USA), a humanized monoclonal antibody against the HER-2 receptor. Overexpression of HER-2 may occur as a somatic genetic change in breast cancer and other tumors. This correlates with poor clinical prognosis and serves as a marker for effective therapy with trastuzumab, either alone or in combination with chemotherapy. Results from a randomized controlled study show that adding trastuzumab to first-line chemotherapy seems to be beneficial in women with metastatic breast cancer that overexpresses HER-2 (Eisenhauer 2001). A FISH test called PathVysion (Abbott Diagnostics) identifies copies of the HER-2 gene in tumor cells. Physicians already use PathVysion extensively for selecting Herceptin patients, and FDA is considering recommendation for changes in labeling of Herceptin to include information about PathVysion to help physicians determine treatment or design clinical trials.

The molecular characterization of childhood leukemias directly affects the treatment strategies. ALL patients whose leukemia lymphoblasts contain the MLL–AF4 or the BCR–ABL fusion are often candidates for allogeneic hematopoietic SCT during first remission. Patients with acute promyelocytic leukemia who carry the PML–RAR alpha fusion respond to all-trans-retinoic acid and have an excellent outcome after treatment with all-trans-retinoic acid in combination with anthracyclines.

6.5.6 Integrated Genome-Wide Analysis of Cancer for Diagnosis and Therapy

An integrated genome-wide analysis of CNV in breast and CRCs using approaches that can reliably detect homozygous deletions and amplifications such as SNP analysis and digital karyotyping has revealed that the number of genes altered by major CNVs, deletion of all copies, or amplification which is at least a dozen copies per cell (Leary et al. 2008). This study has identified genes and cellular pathways affected by both CNVs and point alterations. Pathways enriched for genetic alterations included those controlling cell adhesion, intracellular signaling, DNA topological change, and cell cycle control. A comprehensive picture of genetic alterations in human cancer should therefore include the integration of sequence-based alterations together with copy number gains and losses. Combining copy number and sequence data also holds promise for determining whether particular point mutations have a functional effect, the researchers noted. For example, if a gene turns up with a deletion in one sample and a point mutation in another, it could indicate that that point mutation is inactivating. Incorporating information on other genome-wide changes such as translocations and epigenetic changes could provide even greater insight into cancer, as will trying to determine the timing with which genetic alterations occur in cells. These analyses could prove useful for cancer diagnosis and therapy. For example, two-thirds of the breast and colorectal samples tested in the study

contain alterations to four key signaling pathways, suggesting that drugs targeting these pathways could prove useful for treating both breast and CRCs. Since several breast cancer samples tested contained changes to DNA topological pathways, some of these tumors may be candidates for topoisomerase-targeted therapies.

6.6 Role of NCI in Molecular Diagnosis of Cancer

The role of the NCI in molecular diagnosis of cancer is reflected in the Cancer Genome Anatomy Project. The expanding genetic databases from the Human Genome Project with newly developed expression analysis technologies and holds great promise to help in:

- Understanding the molecular anatomy of normal cells and cells in various stages of disease
- Developing new diagnostic and therapeutic targets for clinical intervention
- Explaining the relationship between genotype and phenotype in humans

NCI has identified various strategic priorities for molecular diagnosis. The institute wants new technologies for early detection of cancer and risk assessment, analytical technologies with potential clinical utility, and technologies for analyzing cancer at the cellular and molecular levels. These could include technologies for genomic, proteomic, or epigenomic biomarker detection technologies; cellular imaging technologies; and POC-related devices such as microfluidics or nanotechnology tools. They also could include "omics" tools for predicting or monitoring patient response, genotyping technologies, high-throughput screening or biosensor technologies, and pharmacogenomic and toxicogenomic tools, among others.

6.7 Future Prospects of Molecular Diagnosis of Cancer

Molecular diagnostic techniques will modify current pathological classifications and grading methods of cancer during the next decade. These will facilitate the identification of molecular targets for the development of new drugs. Gene expression data may help in differentiating primary cancers from metastases. It is anticipated that the histological examination of biopsy sample of a tumor will be supplemented by biochip-based genomic, transcription, and protein analysis to produce a unique tumor fingerprint. Advances in molecular diagnosis of cancer will have a considerable impact on its management.

Diagnostic accuracy is essential for proper management of cancer. Trials of such tests are often reviewed. Systematic reviews are well recognized for enabling efficient integration of current information and providing a basis for rational decision-making in management of patients with cancer. Systematic reviews of diagnostic tests, however, are still developing. Review of the methods and reporting

of systematic reviews of diagnostic tests for cancer published since 1990 reveal that the reliability and clinical relevance of reviews are compromised by poor review methods and reporting. Such reviews require detailed information about the design, conduct, and results of the included primary studies, as well as review methods.

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Chapter 7
Cancer Immunotherapy: Vaccines

7.1 Introduction

Cancer immunology deals with the study of natural interplay between oncogenesis, inflammation, and immune surveillance of the body as well. Immune mechanisms may contribute to the efficacy of some currently used chemotherapeutic agents that may involve recognition of tumor-associated antigens or controlling growth of cancer stem cells. Immunological biomarkers may be used to determine prognosis of cancer and predict the efficacy of anticancer action. Important methods of immunotherapy for cancer are cytokines, monoclonal antibodies (MAbs), vaccines, and immunogene therapy. Following an introduction to cytokines, cancer vaccines will be the focus of this chapter. MAbs will be dealt with in Chap. 8 and immunogene therapy in Chap. 11.

7.2 Cytokines

Cytokines (secreted proteins) are polypeptide hormones secreted by a cell that affect growth and metabolism either of the same cell or of another cell. This definition excludes the hormone produced by various endocrine organs and usually implies signaling molecules produced by the cells of the immune system. The first cytokine to be discovered was interferon. During the last decade, a considerable amount of work has been done in this area, including identification of several of these novel proteins. A number of cytokines have been approved for therapeutic use in humans. The cellular components of the immune system involve a complex network of immune and hematopoietic cells that are alternatively activated and inactivated by cytokines. Various therapeutic strategies based on cytokines have been employed for cancer including cytokine gene therapy. Cytokines are also used as natural adjuvants of vaccines of various formulations to help in activating and maintaining an antitumor immune response.

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_7,
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7.2.1 Interleukins and Cancer

Interleukins are a group of cytokines that were originally observed to be expressed by leukocytes but are now known to be produced by a wide variety of body cells. Interleukins play an important role in the immune system and have been used to treat human cancer. A few examples are given here.

Interleukin (IL)-2, a T-cell growth factor, has been largely used to activate T and NK cells in vivo and to maintain such activation for therapeutic purposes. When given to patients, IL-2 was shown to cause clinical responses, especially in metastatic melanoma and renal cancer patients, though its mechanism of action could not be completely elucidated.

IL-6 is a multifunctional cytokine which plays an important role in a wide range of biologic activities in different types of cells including cancer cells. IL-6 is involved in the host immune defense mechanism as well as the modulation of growth and differentiation in various malignancies. These effects are mediated by several signaling pathways, in particular the signal transducer and transcription activator 3. Deregulated overexpression of IL-6 has been associated with inhibition of cancer cell apoptosis, stimulation of angiogenesis, and drug resistance, which result in progression of cancer. Increased serum IL-6 concentrations in patients are associated with advanced stages of various cancers and reduced length of survival in patients. Therefore, anti-IL-6 therapy for blocking IL-6 signaling is a potential therapeutic strategy for cancer that is characterized by IL-6 overproduction. Preliminary clinical evidence has shown that antibody-targeted IL-6 therapy was well tolerated in cancer patients (Guo et al. 2012).

IL-12, secreted by immune cells in response to an invading pathogen, can also alert T cells to recognize, attack, and remember tumor cells for months to come. IL-12 or its gene is most effective when injected directly into tumors, not infused into the bloodstream. The much higher concentration of IL-12 when applied directly to tumors prompts cells to express specific genes at higher levels and/or transmit signals along different anti-proliferation or suicide-inducing pathways. It also suggests that identifying these specific genes and pathways might enable amplification of their tumor-shrinking effects with IL-12 or other compounds.

IL-15 acts through its specific receptor, IL-15Rα, which is expressed on antigen-presenting dendritic cells, monocytes, and macrophages. IL-15 induces the differentiation and proliferation of T, B, and natural killer (NK) cells. Boosting of IL-15 activity could enhance innate and specific immunity and fight tumors because it does not stimulate immunosuppressive T regulatory cells (Tregs). IL-15 has demonstrated antitumor activity in preclinical models and is a promising agent for anticancer therapy (Steel et al. 2012). Clinical trials of IL-15 in patients with cancer are ongoing.

IL-17A, mainly produced by the T-cell subtype Th17 cells, is an important proinflammatory cytokine that plays a vital pathogenic role in the process of human inflammatory bowel diseases and also has a role in the development of colorectal cancer (CRC). IL-17A is a biomarker and potential therapeutic target in CRC (Shi et al. 2013).

IL-24 (melanoma differentiation-associated gene-7 or mda-7) is classified as an anticancer gene because it selectively induces cell death in cancer cells while having

little or no effect on normal cells. The exact mechanisms by which IL-24 functions are not clear but several pathways have been identified: endoplasmic reticulum stress, ceramide-mediated events, and the generation of reactive oxygen species (Whitaker et al. 2012). Significant progress has been reported regarding the clinical potential of this anticancer gene, and some studies are using mda-7/IL-24 in combination with other cancer therapies.

7.3 Cancer Vaccines

The mechanism for immune surveillance is suppressed in cancer patients. The aim of cancer vaccines is to stimulate the immune system to recognize, attack, and destroy tumor cells. A major difference between infectious diseases and tumors as potential vaccine targets is that cancer cells are derived from the host and consequently evade host recognition as the immune system sees such tumor tissues as "self." The major hurdles in developing cancer vaccines include identification of tumor-specific antigens that focus the extraordinary specificity of the immune system on cancer cells without harming normal cells; development of methods to induce an immune response to eradicate the tumor, in the face of self-tolerance to many tumor antigens; and overcoming mechanisms by which tumors evade the host immune response. The term "cancer vaccines" is somewhat misleading as they do not prevent occurrence of cancer but destroy existing cancers. In contrast to vaccines for prophylaxis of infectious diseases, cancer vaccines are therapeutic. There are several types of cancer vaccines, which include nucleic acid-based, MAb-based, and cell-based vaccines.

7.4 Antigen-Specific Cancer Vaccines

Many cancer vaccines target antigens expressed by cancer cells. The identification of tumor-associated antigens has provided the basis for new concepts in antigen-specific immunotherapy. While much of the success in this area lies with passive immunotherapy, active vaccination strategies are also showing promise. Cancer cells express a wide profile of different proteins, some of which are related to oncogenic transformation and are specific to cancer cells only. However, in cancer, most protein targets presented to the immune system from tumor cells are self-antigens. The immune system is highly tolerant of, and therefore does not react to, these self-antigens. Active immunotherapy aims to reverse this immune tolerance so the immune system can respond appropriately to self-antigens. T helper lymphocytes play an important role in the immune response against cancer (Fig. 7.1). They are able to directly activate antigen-presenting cells to enhance the activity of cytotoxic T lymphocytes (CTLs). T helper cells also secrete cytokines that augment the immune response. Some T helper lymphocytes can induce a direct or indirect killing effect on tumor cells.

Early clinical trials on cancer vaccines were designed to evaluate the toxicity and objectively measurable immunologic effects in relation to clinical developments mostly in patients with metastatic disease. MHC class I- and II-restricted peptide

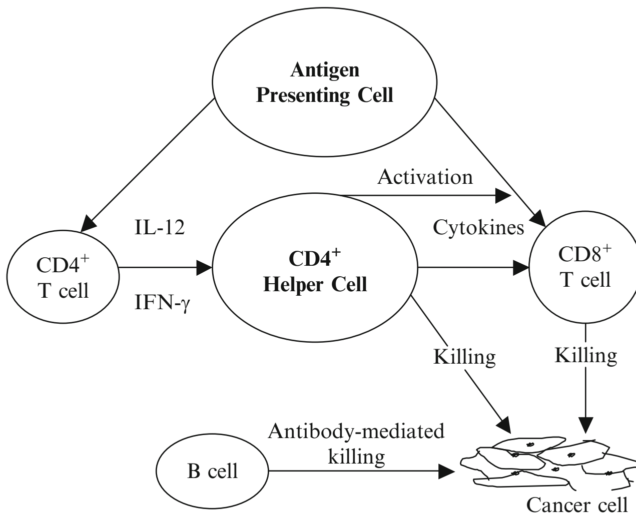

Fig. 7.1 Schematic role of T helper cells in immune response to cancer

epitopes, antigenic proteins, viral constructs, mini-genes, and whole tumor cells have been used either alone or combined with different cytokines (i.e., IL-2, IL-12, GM-CSF), adjuvants (incomplete Freund's adjuvant, montanide, QS21), or dendritic cells to induce specific immune responses in vivo. Standardized assay systems to evaluate the immunologic effects of cancer vaccines have been established and used to study vaccine-induced immune responses during and after vaccination in patients. Prognostic tumor features, i.e., homogeneity of tumor antigen and MHC class I/II expression and intratumoral cellular infiltrates, have been identified that may help to select patients who are more likely to benefit from antigen-specific cancer vaccines in the future.

7.5 Carcinoembryonic Antigen-Based Vaccines

Carcinoembryonic antigen (CEA) is a glycoprotein that is normally expressed in certain parts of the body and commonly overexpressed in most carcinomas of the colon, rectum, breast, lung, pancreas, and gastrointestinal tract. Increased expression of CEA promotes increased intercellular adhesions, which may lead to metastasis. Carcinoembryonic antigen is often used as a serologic marker of malignancy because of its overexpression in cancer as well as its measurability in serum. However, because CEA is normally expressed in the body, the immune system commonly becomes tolerant to it. If this tolerance can be overcome without leading to autoimmune disease, CEA vaccination therapy could be immensely beneficial to cancer patients. A number of preclinical and clinical studies have been conducted on the use of recombinant CEA-vaccinia virus vaccines and recombinant ALVAC-CEA vaccines. In general, the vaccines have been well tolerated and effective at inducing

CEA-specific cytotoxic T-cell responses, especially when used in the presence of the T-cell costimulatory molecule B7.1. "Prime and boost" techniques combining both types of vaccines and the addition of cytokines such as granulocyte–macrophage colony-stimulating factor have resulted in enhanced T-cell responses. The combination of vaccinia or ALVAC vaccines with a triad of costimulatory molecules can also stimulate significant T-cell increases.

7.6 Carbohydrate-Based Cancer Vaccines

Globo H (GH) is a hexasaccharide specifically overexpressed on a variety of cancer cells and, therefore, a good candidate for cancer vaccine development. To identify the optimal carrier and adjuvant combination, GH was chemically synthesized and linked to a carrier protein, including keyhole limpet hemocyanion, diphtheria toxoid (DT) cross-reactive material, and tetanus toxoid, and combined with an adjuvant (Huang et al. 2013). It was administered to mice for the study of immune response. Glycan microarray analysis of the antiserum obtained indicated that the combination of GH-DT adjuvanted with the α-galactosylceramide C34 produced the highest enhancement of anti-GH IgG. Compared with the phase III clinical trial vaccine, GH-keyhole limpet hemocyanion/QS21, the GH-DT/C34 vaccine elicited more IgG antibodies, which are more selective for GH and the GH-related epitopes, stage-specific embryonic antigen 3 (SSEA3) and SSEA4, all of which were specifically overexpressed on breast cancer cells and breast cancer stem cells with SSEA4 at the highest level (>90 %). Therefore, SSEA4-DT/C34 was further developed as a vaccine candidate, and after immunization, it was found that the elicited antibodies are also IgG-dominant and very specific for SSEA4.

7.7 Vaccines Based on Multiple Tumor-Associated Peptides

IMA901 (Immatics Biotechnologies) is the first therapeutic vaccine for renal cell cancer (RCC) consisting of multiple tumor-associated peptides (TUMAPs) confirmed to be naturally presented in human cancer tissue. Human leukocyte antigen A subjects with advanced RCC were treated with IMA901 in two consecutive studies (Walter et al. 2012). In the phase I study, the T-cell responses of the patients to multiple TUMAPs were associated with better disease control and lower numbers of pre-vaccine forkhead box P3 regulatory T cells. The randomized phase II trial showed that a single dose of cyclophosphamide reduced the number of regulatory T cells and confirmed that immune responses to multiple TUMAPs were associated with longer overall survival. Furthermore, among six predefined populations of myeloid-derived suppressor cells, two were prognostic for overall survival, and among over 300 serum biomarkers, the authors identified apolipoprotein A-I and chemokine ligand 17 as being predictive for both immune response to IMA901 and overall survival. A randomized phase III study to determine the clinical benefit of treatment with IMA901 is ongoing.

7.8 Tumor Cell-Based Vaccines

Whole tumor cells, rendered safe by irradiation and mixed with an immunological adjuvant, were one of the earliest forms of cellular therapy. This approach avoids the need for tumor antigens to be identified before treatment and allows all of the relevant antigens to be included in the vaccine. Initial clinical studies showed the safety of this approach, with side effects mainly limited to local reactions at the site of the vaccine injection. Immunogenicity of tumor cell vaccines can be improved by transducing the tumor cell with genes that encode key components of the immune response (cytokines such as granulocyte–macrophage colony-stimulating factor and costimulatory molecules).

Whole tumor vaccines have gone through clinical trials. Melacine® (Corixa Corporation) consists of lysed cells from two human melanoma cell lines combined with a proprietary adjuvant. It is approved for metastatic melanoma in Canada. None of the tumor cell vaccines are in the market in the USA.

7.8.1 BiovaxID

The BiovaxID™ (Accentia BioPharmaceuticals) cancer vaccine evokes the power of each patient's immune system and primes it to recognize and eliminate cancerous lymphoma cells, while sparing normal B cells. In this individualized therapy, cells are harvested from a patient's lymph node, and the unique cancer biomarkers on the outside of their cancer cells are identified. To create this idiotype vaccine, the antigen-bearing tumor cells are fused to antibody-producing mouse cells that act as mini-factories, churning out large quantities of the protein antigens, which are then given back to patients with an immune system booster. By priming the immune system with this antigen in the form of an autologous vaccine, the vaccine induces an immune response against the cancerous cells and creates an immune memory. Because it is derived from individual patient's cancerous cells, the vaccine is a true targeted, personalized therapy. The vaccine's anticancer effect is different from nontargeted traditional therapy, as it arises from the immune system's defense cells' innate ability to selectively target foreign antigens. Moreover, the immune response triggered by the vaccine against the cancerous tissue is a natural disease-fighting mechanism and is associated with minimal toxicity. It is in phase III clinical trials at M. D. Anderson Cancer Center (Houston, TX) for follicular lymphoma, a form of non-Hodgkin lymphoma.

7.8.2 OncoVAX

OncoVAX® (Vaccinogen Inc.) is a vaccine from the patient's own tumor. The cells are dissociated, irradiated to make them non-tumorigenic, and administered to the patient by 3 weekly injections, starting 4 weeks after surgery. A booster vaccination is administered 6 months later. OncoVAX® is administered to patients with colon

cancer after surgery and thereby reduces recurrence and deaths by over 50 %. Vaccinogen is currently preparing to commercialize the vaccine in Switzerland.

7.8.3 Tumor Cells Treated with Dinitrophenyl

An autologous cell vaccine is being developed by AVAX Inc. After removal of a patient's malignant tumor, cancer cells are treated with dinitrophenyl (DNP), a chemical compound known as a hapten, which binds to molecules on the surface of cells and helps trigger immune responses. DNP-treated cancer cells are combined with an adjuvant that enhances their effectiveness and are injected back into the patient. The patient's immune system is then better able to recognize, locate, and combat remaining cancer cells that may have metastasized to other areas of the body. It is these remaining cancer cells that, if left undetected and untreated, can potentially form additional cancerous tumors and eventually lead to death. Immune responses help the body determine which foreign proteins to attack. The ability of DNP to modify proteins and render them more easily identified as foreign to the immune system has been well documented over the past 30 years. AC Vaccine technology applies this same process to cancer cell proteins and other molecules, using the patient's immune system to help prevent recurrence and increase the long-term survival rate.

7.8.4 Vaccines That Simultaneously Target Different Cancer Antigens

Cancer is a difficult disease to tackle because tumor cells mutate over time and a tumor will, therefore, consist of a mixture of slightly different cells. This means that any one marker or antigen may be expressed in some cells and not in others. Onyvax Ltd.'s Cell Vaccines address this problem by simultaneously targeting a broad range of different cancer antigens. These vaccines are made from immortalized cell lines that are representative of different stages of a particular cancer type increasing the likelihood that they will be effective while minimizing side effects associated with many conventional treatments. This approach is particularly effective when little is known of the repertoire of potential targets produced by certain tumor types, such as in the case of prostate cancer. Onyvax lead product is in clinical trials for the treatment of prostate cancer and is poised to enter the final stages of development.

7.8.5 Gene-Modified Cancer Cell Vaccines

A more recent approach is the use of vaccines containing genetically modified cells. The patient's immune system should distinguish between healthy and tumor cells and

recognizes the tumor cells as foreign or diseased. However, sometimes these tumor cells are able to induce tolerance and to escape recognition and destruction by the innate and acquired immune system. The aim of many cell/gene therapy approaches is to design therapeutic vaccines that enable the immune system to recognize and destroy tumor cells, just as it can destroy cells infected with common viral diseases. To remove the tolerance of tumor cells by the patient's immune system, these tumor cells need to be modified by the transfer of genes. These genetically modified tumor cells are then injected into the patients. The most common gene-modified vaccines use cytokines—the cytokine is produced in high concentrations in the vicinity of the tumor cells, where it alters the local immunological environment and enhances the activities of antigen-presenting cells and the activation of tumor-specific T cells. This approach avoids the side effects associated with systemic treatment with cytokines. An example is GVAX vaccine described in the following section.

7.8.5.1 GVAX Cancer Vaccines

Cell Genesys (now acquired by BioSante Pharmaceuticals) was developing GVAX vaccines, which are composed of irradiated tumor cells either from individual patients (autologous) or derived from tumor cell lines which are not patient-specific (allogeneic). These tumor cells have been genetically modified to secrete the immune-stimulating protein granulocyte–macrophage colony-stimulating factor (GM-CSF), enabling the stimulation of an immune response that targets and destroys tumor cells, which persist or recur following surgery and/or radiation therapy. Five phase I/II human clinical trials have been completed in renal cell carcinoma, melanoma, and prostate cancer. An important advantage of GVAX cancer vaccines is that they are "whole-cell" vaccines, which do not require that specific antigens be identified prior to developing the vaccines. It is not known which tumor antigens are most important therapeutically, so Cell Genesys' whole-cell tumor approach utilizes multiple tumor antigens and obviates the need to pinpoint specific antigens to be used in the vaccines. GVAX was reported to show evidence of antitumor activity in humans in all five types of cancer, prostate cancer, pancreatic cancer, lung cancer, melanoma, and kidney cancer suggesting that GVAX may be applicable to multiple types of cancer. A patient-specific form of GVAX was also being explored and is prepared from the patient's own tumor cells and adenoviral gene delivery system in a process that can be completed at the hospital site in 24 h. The future of this product is uncertain although BioSante is seeking future development opportunities for it.

7.8.5.2 K562/GM-CSF

Imatinib mesylate (Gleevec) is one of the first targeted cancer therapies with wide success in CML patients, but it needs to be maintained for the lifetime of the patients. More than 90 % of them achieve remission, but 10–15 % of patients cannot tolerate the drug long term. Often patients have low blood cell counts, fluid

retention, fatigue, significant nausea, and other gastrointestinal problems. Imatinib destroys most leukemic cells in the body, but in most patients, some cancerous cells remain and are measurable with sensitive molecular tests. These remaining cells are a source of relapse, especially if Gleevec therapy is stopped. A pilot study has shown that K562/GM-CSF, a vaccine made from CML cells irradiated to halt their malignant potential and genetically altered to produce GM-CSF, may be able to reduce or eliminate the last remaining cancer cells in some CML patients taking the drug imatinib mesylate (Smith et al. 2010). The treated cells also carry molecules, called antigens, specific to CML cells, which prime the immune system to recognize and kill circulating CML cells. The investigators plan to test blood samples taken from the study patients to identify these antigens to enable them to tailor the vaccine for additional studies that monitor immune response more precisely. Should this vaccine approach prove to be successful, the ability to get patients off lifelong imatinib therapy would be a significant advance.

7.8.6 Active Immunotherapy Based on Antigen Specific to the Tumor

Dendreon Corporation's approach to active immunotherapy development is focused on overcoming the limitations of the immune system and directing it to mount an attack against cancer cells. Activating the immune system begins with the selection and modification of a tumor antigen specific to the cancer (e.g., prostatic acid phosphatase found in ~95 % of prostate cancers), which is produced using recombinant DNA technology. A proprietary technique is then used to isolate antigen-presenting cells taken from a cancer patient, which are combined with the modified antigen using Dendreon's proprietary Antigen Delivery Cassette™. The activated cells are then readministered to the patient to stimulate T cells to recognize and attack cancer cells that contain prostatic acid phosphatase. The lead product in this category is sipuleucel-T (Provenge™), which targets prostatic acid phosphatase. Provenge has been approved by the FDA for the treatment of patients with early-stage and advanced prostate cancer. In clinical studies, patients typically received three infusions over a 1-month period as a complete course of therapy. A phase III clinical trial, IMPACT (IMmunotherapy for Prostate AdenoCarcinoma Treatment) found that Provenge reduced the risk of death by 22.5 % compared with a placebo. The treatment extended the lives of patients by 4–5 months and 33 % of patients with advanced disease were still alive 3 years after treatment with Provenge.

Although Provenge is prostate-specific, the underlying principle may be applicable to other cancers and it may be used in combination with chemotherapy. Dendreon also has several MAbs in preclinical development, which are designed to recognize a specific antigen present on tumor cells but not on healthy cells and bind to that antigen to cause the death of the tumor cell. By this approach healthy cells should not be affected, reducing or eliminating the harsh side effects of many conventional cancer therapies.

7.9 The Use of Dendritic Cells for Cancer Vaccination

Dendritic cells (DCs), named after their long arms, comprise a system of leukocytes widely distributed in all tissues, especially in those that provide an environmental interface. DCs are derived from bone marrow progenitors and circulate in the blood as immature precursors prior to migration into peripheral tissues. Within different tissues, DCs differentiate and become active in the taking up and processing of antigens, and their subsequent presentation on the cell surface linked to major histocompatibility (MHC) molecules. Upon appropriate stimulation, DCs undergo further maturation and migrate to secondary lymphoid tissues where they present antigens to T cells and induce an immune response. Dendritic cells can be derived from CD34+ precursors in response to granulocyte–macrophage colony-stimulating factor and tumor necrosis factor and from monocytes cultured with granulocyte–macrophage colony-stimulating factor and interleukin-4. DCs have the capacity to prime tumor-specific T-cell responses and are considered to be potentially effective vaccines for immunotherapy of cancer.

7.9.1 Autologous Dendritic Cells Loaded Ex Vivo with Telomerase mRNA

Telomerase reverse transcriptase (hTERT) represents an attractive target for cancer immunotherapy because hTERT is reactivated in most human tumors. GRNVAC1 (Geron Corporation) is a therapeutic cancer vaccine comprised of autologous dendritic cells loaded ex vivo with hTERT mRNA. Results of the first completed phase I/II clinical trial of GRNVAC1 in metastatic prostate cancer patients showed that the vaccine was well tolerated with no major treatment-related toxicities (Su et al. 2005). In addition, telomerase-specific T-cell responses were generated in 19 of 20 subjects, and vaccination was associated with a statistically significant increase in PSA doubling time and clearance of prostate cancer cells from the patients' blood, indicative of potential clinical response. The telomerase vaccine is currently in multiple phase I/II trials at Duke University (Durham, NC) where different strategies to optimize vaccine performance are under evaluation.

7.9.2 Dendritic Cell-Targeted Protein Vaccines

Most of current vaccines primarily work by inducing protective antibodies, but there is a need for durable and protective T-cell immunity. A safe T-cell-based protein vaccine is being developed that exploits the pivotal role of DCs in initiating adaptive immunity (Trumpfheller et al. 2012). Focusing on HIV, gag-p24 protein antigen is introduced into an MAb that efficiently and specifically targets the DEC-205 antigen uptake receptor on DC. When administered together with synthetic dsRNA, polyriboinosinic–polyribocytidylic acid, or its analog stabilized with carboxymethylcellulose

and poly-L-lysine as adjuvant, HIV gag-p24 within anti-DEC-205 MAb is highly immunogenic in mice, rhesus macaques, and healthy human volunteers. Human subjects form both T-cell and B-cell responses to DC-targeted protein. Thus, DC-targeted protein vaccines are a potential platform, either alone or in combination with highly attenuated viral vectors, for inducing integrated immune responses against microbial or cancer antigens, with improved ease of manufacturing and clinical use.

7.9.3 Dendritic/Tumor Cell Fusion

Dendritic/tumor cell fusion is used to create a patient-specific cancer vaccine. The technology combines a patient's dendritic cells with their inactivated tumor cells using a chemical fusion or an electrofusion procedure. The fused cells are injected back into the patient in order to stimulate an immune response against the patient's cancer. Cell fusion technology eliminates the need to identify the appropriate specific antigens to create a tumor vaccine because it incorporates the entire menu of antigens found on the original tumor to provide the target for the immune system. Once administered, a cancer vaccine stimulates the immune system to seek out and destroy cancer cells that display the antigens included in the vaccine. Antigens, protein fragments that are present in cancer cells, function as markers to direct an immune response to those cells. Because cancer vaccines elicit a systemic immune response, they have the potential to destroy cancer wherever it is found in the body.

Immunization with hybrids of DCs and tumor cells has also been shown to produce protective immunity and rejection of established tumors in various rodent models. A high-throughput electrofusion technique can provide efficient fusion rates in a variety of murine and human tumor cell lines. The fused cells display a mature DC phenotype and express tumor-associated antigens. Phase I/II clinical trials in patients with progressive metastatic renal cell carcinoma using DC precursor cells of healthy donors fused with either allogeneic or autologous renal tumor cells showed an increase in the reactivity against recall antigens in most patients and increased cytotoxicity of peripheral blood lymphocytes against renal cell carcinoma during treatment as well as rise in the percentage of interferon-γ-secreting cells. The therapeutic efficacy of a DC–tumor fusion vaccine is now being evaluated for the treatment of metastatic melanoma.

7.9.4 Genetically Modified Dendritic Cells

In vitro generated DCs, which have been transduced with genes coding for tumor-specific antigens or pulsed with tumor-specific antigen or peptide, could be useful for induction of cytotoxic T-cell responses. Genetic engineering of DCs to express immunosuppressive or immunoregulatory molecules may provide a novel method to promote graft tolerance, reducing dependence on systemic immunosuppression.

Gene therapy techniques can be applied to DC vaccines using recombinant non-replicating viral vectors to provide efficient and reliable means of gene transfer.

Genetic material is introduced into DCs to provide them a renewable source of antigen for presentation; this should lead to more sustained expression of antigen. The expression of viral (and therefore foreign) genes may boost the immune response, but this antiviral immunity primed by DCs may cause the immune system to destroy DCs rapidly in subsequent rounds of immunization. One solution may be to use viral vectors that do not result in the expression of viral genes, such as retroviruses or "gutless" adenoviral vectors. Adeno-associated viruses can be used to transduce human DCs and their main advantage is a decrease in viral-derived epitopes leading to decreased immunogenicity of the vector.

Lentivirus vectors can be used for genetic modification of human DCs and they have an advantage over retroviral vectors that they do not require target replication for efficient transduction. Approaches facilitating generation of DC vaccines for clinical trials and enhancing their viability, biodistribution, and capacity to stimulate antigen-specific immune responses are critical for immunotherapy. In one study, mouse bone marrow cells were programmed with lentiviral vectors so that they produced GM-CSF and IL-4 in an autonomous manner (Koya et al. 2007). Mice vaccinated with genetically modified DCs self-differentiated in vitro or in vivo and produced potent antigen-specific responses against melanoma, which correlated with protective and long-term therapeutic antitumor effects. Thus, DC precursors can be genetically engineered after a single ex vivo manipulation, resulting in DC vaccines with improved activity.

7.9.5 Preclinical and Clinical Studies with DC Vaccines

DCs are the most potent antigen-presenting cells and the only ones capable of presenting novel antigens to naive T cells. Large numbers of DCs can be generated in vitro in the presence of appropriate cytokine cocktails using either adherent peripheral blood mononuclear cells or CD34+ precursors. DC preparations for cancer are:

- Blood DCs—density gradient separated and immunoselected (CMRF44+, CMRF56+)
- Mononuclear DCs—adherent mononuclear cells and immunoselected (CD14+)
- Stem cell-derived DCs—immunoselected (CD34+)
- DC-derived exosomes

Several preclinical studies have demonstrated the effectiveness of antigen-loaded DC to mediate antitumor immune responses. Clinical trials have shown DC as a promising tool for the immunotherapy of cancer. In these trials, white blood cells are removed from the patient and are treated ex vivo with cytokines for several days and the resulting DCs are fed with cancer antigen just prior to reinfusion into the patient.

Stimuvax® (Biomira Inc.) vaccine, based on technology licensed from Cancer Vac Ltd., is in a phase III randomized clinical trial of patients with metastatic NSCLC to establish the safety profile and determine whether an immune response

against the vaccine translates into clinical survival benefit. The basis of this vaccine is that potent primary anti-MUC1 T-cell responses could be generated in vitro following incubation of human dendritic cells with liposomes followed by the addition of autologous T cells to the culture.

The Center for Cell and Gene Therapy of the Baylor College of Medicine (Houston, TX) is using Ampligen (Hemispherx Biopharma Inc.), a phase III experimental drug that stimulates the immune system, to substitute for multiple other cytokines as the DC maturing agent. DCs thus matured will be used in a clinical trial on patients with a relapsed form of Hodgkin lymphoma.

A phase I study on patients with metastatic melanoma at Duke University (Durham, NC) is assessing the safety as well as efficacy of vaccination with melanoma tumor-associated antigen-encoding RNA-transfected mature DCs derived from monocytes that have been either untreated, transfected with control siRNA, or transfected with siRNA targeting the inducible immunoproteasome beta subunits. A combination of RNAs encoding melanoma tumor-associated antigens is administered by intradermal injection.

7.9.6 Vaccines Based on Dendritic Cell-Derived Exosomes

Exosomes are nanovesicles originating from late endosomal compartments and secreted by most living cells in ex vivo cell culture conditions. B-cell and DC-derived exosomes can mediate MHC-dependent immune responses, which have clinical applications in cancer. Preclinical studies have shown immunogenicity of DC-derived exosomes (Dex) and vaccination trials using Dex strategy have been conducted in melanoma and NSCLC patients (Viaud et al. 2010).

7.9.7 Limitations of DC Vaccines for Cancer

Although DCs have directly induced immunity in many clinical trials, only limited success has been achieved in terms of inducing partial or complete remissions in cancer patients. In many cases DCs used in the treatment of patients with cancer are differentiated in vitro from blood monocytes and activated in vitro by cytokines and PGE2, which render them resistant to in vivo licensing by costimulatory molecules such as CD40 (Gilboa 2007). These DCs fail to induce cytokines such as IL-12 that skew the immune response to a Th1 response. DC function might be further improved by inhibiting negative regulatory pathways in the cells or by inducing the cells to express costimulatory molecules and antiapoptotic proteins to enhance viability. Improving the delivery of antigens to DCs is also required. Although several vehicles have been used for this purpose (viral vectors and apoptotic or necrotic tumor lysates), there is no consensus on the optimal approach.

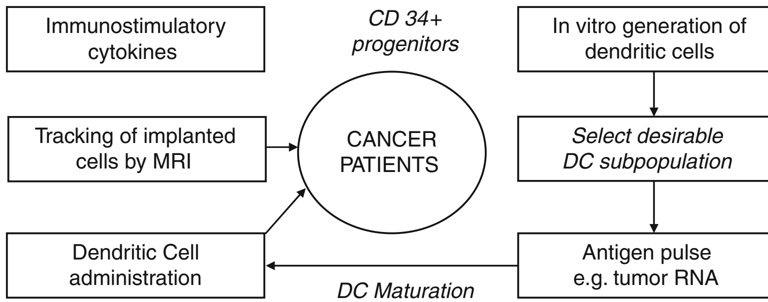

Fig. 7.2 A scheme of generation and administration of tumor antigen-pulsed dendritic cells

7.9.8 *Future Developments to Enhance Clinical Efficacy of DC Vaccines*

Recent advances in the basic molecular understanding of positive and negative regulation of antigen presentation and immune responses can form a basis to enhance the efficacy of DC-based vaccines. Mobilization of a large number of DCs or their precursors and automation of the culture process may increase the efficiency of generating DC vaccines. Combining the powerful new tools of immunomonitoring with improved dendritic cell vaccination protocols will enable rationally designed scientific studies to give cancer immunotherapy further credibility and cancer patients a new therapeutic option. Strategies for increasing the efficacy of tumor antigen-pulsed DCs are shown in Fig. 7.2.

An understanding of Toll-like receptor, tumor necrosis factor receptor, and cytokine receptor signaling in activation of innate and adaptive immunity is important. DC vaccines seem to have the best potential of all other cancer vaccines. Future DC-based therapies will include monoclonal antibodies (MAbs), genetic modification of DC, the use of CD34+ precursors, direct delivery of DC to tumors, and application of tumor lysates or apoptotic cells as sources of additional, as yet undefined, antigens.

Loading of DC with anti-MHC class I chain-related protein A MAb-coated cancer cells efficiently promotes tumor antigen cross-presentation and priming of multivalent antitumor CD8 and CD4 T-cell responses. These are of substantially greater breadth and magnitude than those of T cells primed by peptide-pulsed or apoptotic tumor cell-loaded DCs. These results may advance DC vaccine development and provide a platform for adoptive T-cell therapy and tumor antigen discovery.

To enhance loading of DCs with oncoproteins in vitro and to increase the efficacy of the vaccines, a variety of genetic manipulations have been proposed and shown to be efficient in experimental tumor models. DCs were transfected either with polynucleotides, DNA or RNA, coding for tumor-associated antigens or with DNA encoding immunostimulatory cytokines and costimulatory molecules. The delivery of genes coding for antigenic epitopes or other molecules with a

recombinant retrovirus, adenovirus, or poxvirus into dendritic cells has also been used for transduction and therapy. As an alternative method for tumor-associated antigen delivery into DCs, fusion of DCs with tumor cells has been utilized and the hybrid cell-based vaccines have been found to be highly active in cancer patients.

Current DC vaccine preparations involving ex vivo differentiation and maturation produce short-lived, transiently active DCs that may curtail T-cell responses in vivo. Akt1, downregulation of which decreases DC life span, is critical for proinflammatory signal-mediated DC survival and maturation. Lipopolysaccharide or CD40 signaling stabilizes Akt1, promoting both activation and bcl-2-dependent survival of DCs. Expression of a potent allele encoding a lipid raft-targeted Akt1, MF-DeltaAkt, is sufficient for maturation and survival of murine bone marrow-derived DCs in vivo. MF-DeltaAkt-transduced DCs enhance T-cell proliferation, activation, and long-term memory responses, enabling eradication of large preestablished lymphomas and aggressive B16 melanomas. Human myeloid DCs expressing constitutively active MF-DeltaAkt also survive significantly longer and promoted antigen-specific T-cell responses. Thus, Akt1 is a critical regulator of DC life span, and its manipulation in DCs can improve the clinical efficacy of DC-based tumor vaccines.

DCs are usually produced by culturing monocytes in the presence of IL-4 and GM-CSF for 5–7 days (Standard DC), but a modified protocol is available for differentiation of human monocytes into mature DCs within 48 h (Fast DC). A functional comparison of the two methods shows that mature Fast DCs are functional APCs capable of inducing primary T-cell responses and suggests that these cells may be valuable for generation of anticancer vaccines (Jarnjak-Jankovic et al. 2007).

Tracking the fate of DCs is important. Cell Sense technology (Celsense Inc.) has been used to image transplanted cells by MRI in a phase I clinical trial of an autologous dendritic cell vaccine to treat colorectal cancer at University of Pittsburgh Cancer Institute.

7.10 Lymphocyte-Based Cancer Therapies

The immune system has basically two types of lymphocytes: killer T lymphocytes eliminate tumors or virally infected cells, and helper T lymphocytes help to activate killer T lymphocytes. T cells play a critical role in fighting infection and cancer. Several anticancer therapies are based on these cells.

7.10.1 Adoptive Cell Therapy

Adoptive cell therapy (ACT), also called adoptive immunotherapy, is the isolation of antigen-specific T lymphocytes, their ex vivo expansion and activation, and subsequent administration in large numbers to the autologous host. It is a promising approach to inducing antitumor immune responses. The molecular identification of

tumor antigens and the ability to monitor the persistence and transport of transferred cells have provided new insights into the mechanisms of tumor immunotherapy. Several studies have shown the effectiveness of ACT for the treatment of patients with selected metastatic cancers. Important features of studies on this topic are as follows:

- Preclinical models have identified characteristics of lymphocyte cultures that are required for successful ACT therapy.
- The most important characteristic is the presence of high affinity, tumor antigen-specific CD8+ T cells. There is generally a direct correlation between treatment efficacy and the number of transferred, tumor-specific cells.
- Ways to manipulate the host immune environment that increase ACT therapeutic efficacy as identified in preclinical models include immunosuppression before cell administration and concurrent IL-2 administration with the transferred T cells.
- Lymphocyte cultures that were selected for reactivity against melanoma antigens, including melanocyte-differentiation antigens, mediated cancer regression in some patients with metastatic melanoma. Melanoma-reactive cultures that were suitable for ACT therapy were generated from tumor-infiltrating lymphocytes that were rapidly expanded with anti-CD3 antibody.
- The generation of tumor antigen-specific lymphocyte cultures is evolving rapidly, spurred on by the identification of tumor antigens and the T-cell receptors that recognize them.
- Further improvements to ACT therapy will depend on a deeper understanding of basic immunological processes, including the role of CD4+ T cells in the antitumor inflammatory response, the ability of lymphocytes to persist in vivo and travel to tumors, and the mechanisms of ACT augmentation by previous host immunosuppression.

ACT regimen results in the in vivo expansion and enhanced activity of these cytotoxic lymphocytes. NCI has identified and characterized a number of melanoma tumor-associated antigens, including gp100 and MART-1, and has developed a lymphodepleting nonmyeloablative regimen used for ACT. Favorable results were seen in phase II clinical trials. Transgene and the NCI have collaborated to evaluate new candidate cancer vaccines, with the objective to assess the boosting effect of the vaccination on the lymphocytes' activity. The adoptive transfer of in vitro generated tumor antigen-specific cytotoxic T lymphocytes (CTL) provides a promising approach to the immunotherapy of cancer. A phase I study was conducted to test the feasibility, safety, and survival of adoptively transferred Melan-A-specific CTL lines in melanoma patients and shown to induce clinical tumor-specific immune responses without major adverse effects (Mackensen et al. 2006). A phase II trial showed that all the clinical responses were significantly associated with in vivo expansion of the Melan-A-specific T-cell repertoire (Khammari et al. 2009). Thus, over the course of an ACT, monitoring this melanoma-specific T-cell expansion in patient blood appears crucial for predicting the clinical efficiency of such an immunological approach.

Tumor infiltrating lymphocytes (TILs) already appear to offer significant patient benefit and this approach now warrants further development. Genetically engineered T cells offer a means to endow peripheral blood T cells with antitumor

activity, and in principle these techniques could allow the treatment of a wide range of cancers. Genetic engineering also offers the means to endow T cells with new properties and enhanced functions. There have been clear proof-of-principle trials showing responses with T-cell receptor (TCR)-engineered T cells and this can be developed further (Hawkins et al. 2010).

Epstein–Barr virus (EBV) infection is associated with a heterogeneous group of tumors, including lymphoproliferative disorders, Hodgkin disease, nasopharyngeal carcinoma, and Burkitt's lymphoma. As such neoplastic disorders express viral antigens, they can be treated by ACT relying on in vitro generation and expansion of virus-specific CTLs, which can be administered to patients for both prophylaxis and treatment. ACT with EBV-specific CTL is safe, well-tolerated, and effective in the case of most immunogenic tumors such as posttransplant lymphoproliferative disease (Merlo et al. 2008).

ACT could play a role in cancer treatment if infused cells can be quickly eliminated in case of adverse events. A safety switch has been constructed by introducing the gene into donor T cells given to enhance immune reconstitution in recipients of haploidentical stem cell transplants. AP1903, a dimerizing drug, given to four patients in whom GVHD developed, eliminated more than 90 % of the modified T cells within 30 min after administration and ended the GVHD without recurrence (Di Stasi et al. 2011).

7.10.2 Chimeric Antigen Receptor T Cells

Chimeric antigen receptors (CARs) combine the antigen-binding site of an MAb with the signal activating machinery of a T cell, freeing antigen recognition from MHC restriction and thus breaking one of the barriers to more widespread application of cell therapy.

Human T cells targeted to the B-cell-specific CD19 antigen through retroviral-mediated transfer of a CAR called 19z1 have shown significant but partial in vivo antitumor efficacy in an acute lymphocytic leukemia (ALL) model (Brentjens et al. 2007). The causes of treatment failure in this model were investigated and approaches were designed to enhance the efficacy of this adoptive strategy. Expression of the 19-28z CAR, containing the signaling domain of the CD28 receptor, enhanced systemic T-cell antitumor activity when compared with 19z1 in treated mice. T-cell injections, designed to prolong in vivo T-cell function, further improved long-term survival. Thus combined in vivo costimulation and repeated administration enhance eradication of systemic tumor by genetically targeted T cells. The finding that modifications in CAR design as well as T-cell dosing enable the complete eradication of systemic disease affects the design of clinical trials using this treatment strategy. The idea is that a patient's own T cells are taken and reeducated by inserting a gene into them that will enable them to produce a receptor to recognize B-cell cancers, and then they are returned to the patient where they should be able to attack and kill the tumor cells. Because the technique uses a patient's own T cells, there are no incompatibility issues or rejection, as there might be with human stem cell transplant.

T cells bearing CARs kill cells expressing target antigens on their surface, in a human leukocyte antigen (HLA)-independent manner. T cells expressing CARs are highly targeted like MAbs, but also offer the potential benefits of active trafficking to tumor sites, in vivo expansion, and long-term persistence. Furthermore, gene transfer in T cells enables the introduction of countermeasures to tumor immune evasion and of safety mechanisms (Ramos and Dotti 2011). In another study, a small number of T cells were removed from three patients with advanced-stage CLL, infected with a viral vector (Modified HIV), then expanded in cell culture, and reinfused into the patients (Kalos et al. 2011). Engineered T cells expanded >1,000-fold in vivo, trafficked to bone marrow, and continued to express functional CARs at high levels for at least 6 months accompanied by complete remission, in two of three patients. Moreover, a portion of these cells persisted as memory CAR+ T cells and retained anti-CD19 effector functionality, indicating the potential of this MHC-independent approach for the effective treatment of B-cell malignancies. CAR-modified T cells are likely to play an increasing role in cell therapy of cancer. The new genes program the T cells to attack B cells, a normal part of the immune system that turn malignant in leukemia. In patients with lasting remissions after the treatment, the altered T cells persist in the bloodstream, though in smaller numbers than when they were fighting the disease. Some patients have had the cells for years. CAR-modified T cells can kill even aggressive, treatment-refractory ALL cells in vivo, but the emergence of tumor cells that no longer express the target indicates a need to target other molecules in addition to CD19 in some patients with ALL (Grupp et al. 2013).

The treatments are expensive as producing engineered T cells costs about $20,000 per patient, but it is far less than the cost of a bone marrow transplant. Scaling up the procedure should make it even less expensive. Novartis has committed $20 million to build a research center at University of Pennsylvania where the treatment was initially developed to bring it to market.

Adults with relapsed B-cell ALL have a dismal prognosis. Only those patients able to achieve a second remission with no minimal residual disease (MRD) have a hope for long-term survival in the context of a subsequent allogeneic hematopoietic SCT (allo-HSCT). Five relapsed B-ALL subjects were treated with autologous T cells expressing a CD19-specific CD28/CD3 second-generation dual-signaling CAR termed 19-28z (Brentjens et al. 2013). All patients with persistent morphological disease or MRD+ disease upon T-cell infusion demonstrated rapid tumor eradication and achieved MRD− complete remissions as assessed by deep sequencing PCR. Therapy was well tolerated, although significant cytokine elevations, specifically observed in those patients with morphologic evidence of disease at the time of treatment, required lymphotoxic steroid therapy to ameliorate cytokine-mediated toxicities. Cytokine elevations directly correlated to tumor burden at the time of CAR-modified T-cell infusions. Tumor cells from one patient with relapsed disease after CAR-modified T-cell therapy, who was ineligible for additional allo-HSCT or T-cell therapy, exhibited persistent expression of CD19 and sensitivity to autologous 19-28z T-cell-mediated cytotoxicity, which suggests potential clinical benefit of additional CAR-modified T-cell infusions. These results demonstrate the marked antitumor efficacy of 19-28z CAR-modified T cells in patients with relapsed/refractory B-ALL and the reliability of this therapy to induce profound molecular remissions,

forming a highly effective bridge to potentially curative therapy with subsequent allo-HSCT. T cells are still experimental, whereas allo-HSCT is the standard of care in ALL because they have been shown to give many patients the best odds of survival. The study is continuing, and as more patients are treated, answers may emerge as to whether the T cells alone will be enough for some patients with allo-HSCT.

The extensive exploitation of the antitumor effect of donor lymphocytes infused after allo-HSCT is limited by the risk of GVHD. To overcome this limitation, the therapeutic potential of donor lymphocytes engineered with the suicide gene thymidine kinase (TK) of HSV was investigated in patients experiencing recurrence of hematologic malignancies after allo-HSCT (Ciceri et al. 2007). The antitumor effect tightly correlated with the in vivo expansion of TK+ cells. Immunization against HSV-tk was observed in some patients but did not preclude an effective GvL. These data validate the feasibility, safety, and efficacy of TK+ cells in the context of allografting and represent the basis for a broader application of this technology. This technology is being clinically developed by MolMed.

7.10.3 Combination of Antiangiogenic Agents with ACT

Although ACT-based immunotherapies can achieve cancer regression in animal models and in up to 70 % of patients with metastatic melanoma, it is possible that the tumor vasculature impedes the egress of tumor-specific T cells, thus hindering immunotherapy. Disruption of the proangiogenic interaction of VEGF with its receptor VEGFR-2 has been reported to “normalize” tumor vasculature, enhancing the efficacy of chemotherapeutic agents by increasing their delivery to the tumor interstitium. Administration of an antibody against mouse VEGF synergized with ACT to enhance inhibition of established, vascularized, B16 melanoma and improved survival (Shrimali et al. 2010). Anti-VEGF antibody significantly increased infiltration of transferred cells into the tumor. Thus, normalization of tumor vasculature through disruption of the VEGF/VEGFR-2 axis can increase extravasation of adoptively transferred T cells into the tumor and improve ACT-based immunotherapy. These studies provide a rationale for the exploration of combining antiangiogenic agents with ACT for the treatment of patients with cancer.

7.10.4 Expansion of Antigen-Specific Cytotoxic T Lymphocytes

The ex vivo expansion of human cytotoxic T lymphocytes (CTLs) has potential for use in immunotherapy applications for cancer. It is limited by difficulty in obtaining sufficient numbers of CTLs. The Rapid Expansion Method (REM) for antigen-specific CTL has been developed by CellExSys. It enables single-cell antigen-specific CTL expansion by a factor of 10^9 to 10^{10} and can produce therapeutic levels of patient-specific, antigen-specific cytotoxic T lymphocytes. CTLs retain functionality, including antigen-induced proliferation, cytotoxic activity, cytokine release, and memory cell generation. The safety has been tested in studies in patients with

malignant melanoma as well as viral infections such as CMV and EBV occurring after organ transplantation and immunosuppression.

Processing of T cells for clinical trials must be performed according to the principles of cGMP to ensure the identity, purity, potency, and safety of the cellular product. Production of genetically modified T cells for clinical trials requires characterization by cell surface marker phenotype, testing of functional activity against CD19+ targets, and requisite safety testing.

7.10.5 Genetic Engineering of Tumor Cells to Activate T Helper Cells

Many companies have effective vaccines for stimulating killer T lymphocytes. The missing link is making good vaccines for helper T lymphocytes. That problem has been solved by Antigen Express (a subsidiary of Generex Biotechnology Corporation) scientists, who developed means to suppress the expression of a specific immunoregulatory protein (Ii). This protein can block antigens from stimulating T helper cells. By inhibiting this protein, a whole range of antigens from tumors or virally infected cells can now be recognized by T helper cells, greatly boosting the immune response to cancer and pathogenic viruses.

Tumor cells engineered by gene transduction to be MHC Class II+/Ii- are novel APCs capable of presenting endogenous tumor antigen epitopes to activate T helper cells. The MHC Class II+/Ii- tumor cell phenotype is created by transfecting genes for either CIITA or IFN-gamma and inhibiting induced Ii mRNA by an Ii reverse gene construct (Ii-RGC). Adenoviral vectors are preferred for the delivery of such genes because of high transfection efficiency and ubiquity of the adenoviral receptor on many cell types and tumors. A single recombinant adenovirus with both genes for IFN-γ and Ii-RGC (rAV/IFN-γ/Ii-RGC) can efficiently induce the MHC Class II+/Ii- phenotype. Injection of tumor nodules in experimental animals with rAV/Ii-RGC and rAV/CIITA/IFN-γ combined with a suboptimal dose of rAV/IL-2 induces a potent antitumor immune response. These methods are adaptable for producing enhanced genetic vaccines, attenuated virus vaccines (e.g., vaccinia), and ex vivo cell-based vaccines (dendritic and tumor cells).

7.10.6 Rescue of CD8+ T Cells for Use in Tumor Immunotherapy

CD8+ T cells that can recognize tumor antigens but evade thymic deletion are potentially harmful and thus are held in check inside the body by mechanisms that make them tolerant of the protein even if it is encountered on a tumor cell. However, at times the system operates too well. Because tumor cells express higher levels of many of these antigens than do normal cells, some T cells can recognize the tumor cells and largely ignore the normal cells, but these CD8+ T cells are also held in check inside the body by mechanisms that build up their tolerance to the presence

of tumor antigens. They become deficient in sending the signals that lead to tumor cell killing. However, the cells can be rescued from this tolerant state and encouraged to proliferate in vitro if they are mixed with IL-15. The cells are naturally exposed to lower doses of IL-15 inside the body, and this probably helps keep the cells alive despite their tolerant state. However, once these cells are induced to proliferate, they can be expanded to large numbers and are no longer tolerant of the tumor antigen. The expanded tumor-reactive T cells are now effective in treating a disseminated form of leukemia in mice without damaging their livers. This suggests that the liver and other normal tissues expressing lower levels of the antigen may have their own protective mechanisms. Thus CD8+ T cells can potentially be rescued and expanded for use in tumor immunotherapy.

7.10.7 Tumor-Infiltrating Lymphocytes

Tumor-infiltrating lymphocytes (TILs) are lymphoid cells that infiltrate solid tumors. In vivo studies have shown that TILs were capable of homing to tumor site after infusion and persisted for relatively long periods of time. Immunomodulation of TIL function by various cytokines can be performed in vivo. It is also possible to study various subpopulations of TILs such as CD34+ and CD8+. Before using NeoR (neomycin resistance) as a gene marker to conduct studies in humans, many studies were performed either in animals or in human cells in vitro. The NeoR-marked TIL study was the first clinical protocol approved to administer modified genetic material to patients (Rosenberg et al. 1990).

TILs, retrovirally transduced with ScFv-y genes derived from either anti-TNP (trinitrophenol) or antiovarian MAb, were redirected to specifically lyse TNP-labeled cells or ovarian carcinoma cells, respectively. In addition, redirected TILs can secrete granulocyte–macrophage colony-stimulating factor (GM-CSF) following stimulation by the appropriate antigen. These studies indicate that T cells can be redirected to lyse new targets in MHC-independent fashion.

In conclusion, using replication-incompetent human retroviral vectors, the gene marking method appears to be a safe and sensitive technique when combined with PCR to monitor TILs after infusion. TILS can persist in tumor cells for up to a few months. Information gained from the initial studies has led to the development of more complex gene marking protocols, which are intended to overcome the limitation of earlier studies and to extend the range of questions that can be answered, by gene marking.

7.10.8 Hybrid Cell Vaccination

The hybrid cell vaccination approach to cancer immune therapy aims at the induction of tumor-specific cytotoxic T cells and was developed for the following purposes:

- To recruit and activate T-cell help for the induction of tumor-specific cytotoxic T cells

- To correct defects in costimulatory signaling
- To utilize a large number of unidentified tumor-associated antigens
- For individualized therapy that can be applied instantly without long preparation

Hybridoma technology involves selection of long-term lines on the basis of their resistance to anticancer drugs and according to specific functions desired. The fusion partners are of the same tissue origin and are controlled by similar genetic programs. The vaccines are irradiated prior to inoculation to ensure that the tumor does not grow and spread in the body.

Clinical trials of hybrid cell vaccination have been performed in patients suffering from malignant melanoma or renal cell carcinoma and cases of complete remission have been reported. The side effects seen in these trials were those of induced immune response. Hybrid cell vaccination is a feasible strategy for the treatment of cancer and is well suited for individualized therapy. Future trials will establish the criteria for selection of patients and the malignancies suitable for this therapy.

7.11 Chemoimmunotherapy

Chemoimmunotherapy is enhancement of chemotherapy-induced T-cell responses by giving a nonspecific immunostimulatory factor which induces the APCs to mature and transport the tumor antigens to the lymph nodes for presentation to T cells. Preclinical studies have provided evidence for synergy between chemotherapy and immunotherapy in cancer. A phase I/II study has demonstrated that a combination of paclitaxel plus subcutaneous injections of antigen presenting cell (APC) agonist IMP321 (Immutep SA) achieved clinical benefit in 90 % of metastatic breast carcinoma patients at 6 months (Brignone et al. 2010). There was a sustained increase in the number and activation of monocytes and DCs as well as a higher count of NK and long-lived cytotoxic effector-memory CD8+ T cells. Also, the objective tumor response rate of 50 % compared favorably to the 25 % rate reported in the historical control group. The absence of toxicity and the demonstration of activity strongly support the further development of this combination as first-line regimen for cancer.

7.12 Concluding Remarks About Cancer Vaccines

Although no therapeutic cancer vaccine has yet been approved by the FDA, several new paradigms are emerging from the use of combination therapy approaches and clinical trial design. Review of clinical trials involving several different cancer vaccines reveals data contrasting classic tumor response criteria with patient response in the manifestation of increased patient survival following vaccine therapy. There are several strategies in which cancer vaccines can be exploited in combination with other agents and therapeutic modalities that are quite unique when compared with

conventional combination therapies. This is most likely due to the phenomena that (a) cancer vaccines initiate a dynamic immune process that can be exploited in subsequent therapies and (b) both radiation and certain chemotherapeutic agents have been shown to alter the phenotype of tumor cells as to render them more susceptible to T-cell-mediated killing. Even though the tumor may not shrink in response to vaccine therapy, evidence is emerging that patients who receive these vaccines (as contrasted with control cohorts) in trials tend to live longer and respond better to subsequent treatment. An overview of various types of cancer vaccines that can be personalized, their mechanism of action, and current status of development are reviewed elsewhere (Jain 2010).

7.13 Cancer Vaccine Consortium

The Sabin Vaccine Institute (New Canaan, CT) has launched the Cancer Vaccine Consortium to bring together public and private companies that are interested in developing vaccines for cancer. The aim of the consortium is to accelerate the progression of cancer vaccines from R & D into the clinic. Twenty companies have so far joined the consortium and these include GlaxoSmithKline, Pfizer, Antigenics, Coley Pharmaceutical Group, Dendreon, Favrille, StressGen Biotechnologies, BioVex, Cell Genesys, EMD Pharmaceuticals, Igeneon, Northwest Biotherapeutics, Shire Biologics, and Therion Biologics.

Collectively, these companies are conducting clinical trials that target solid tumors of the brain, prostate, skin, breast, lung, cervix, colon, and ovaries as well as hematological cancers. A key role for the consortium members will be to work with regulatory agencies on novel vaccines and other biotherapeutic agents to speed the approval process.

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Chapter 8
Monoclonal Antibodies and Cancer

8.1 Introduction

A monoclonal antibody (MAb) is an antibody made from a single clone (hybridoma) of white blood cells. MAbs are used to block key receptors on tumor cell surfaces, compromising their function. MAbs may also be used to recruit the cellular arm of the immune system, planting a homing beacon on the transformed cell. MAbs, although produced in the laboratory, mimic the antibodies naturally produced by the body as part of immune system's response to disease. The remarkable specificity of MAbs is also being harnessed in other ways, e.g., they are being paired with powerful toxins to create specific agents that seek out cancer cells and kill them. MAbs are also being employed for diagnosis, helping to identify the source of a tumor and provide a possibility for the treatment. MAbs enable the combination of diagnostics with therapeutics.

8.1.1 Murine MAbs

Murine MAbs against tumor-specific antigens were initially envisioned as therapeutic agents capable of directly attacking cancer cells. Once thought to be "magic bullets," it was hoped that murine MAbs would effectively target disease but not affect healthy cells. This approach, however, proved to be disappointing. Since tumors are not well perfused, relatively large doses of the MAbs were required, which caused problems relating primarily to adverse immunological reactions against the antibodies that the body recognized as large foreign proteins. The MAbs of the first generation caused toxicity with little efficacy. Adverse effects of murine MAbs, in combination with poor target selection due to the lack of data on tumor antigens, eventually led to their abandonment as therapeutic agents. Many of the murine MAbs in use now are humanized.

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_8,
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8.1.2 Humanized MAbs

The ability to make MAbs against antigens expressed by human cells has facilitated the identification not only of lymphocytes with different subset functions but also of different antigens on tumor cells. MAbs have several potential targets for cancer therapy, including the CD20 antigen, the IL-2 receptor (CD25), the epidermal growth factor receptor (EGFR), and the VEGF. The FDA has approved several MAbs against cancer listed in Table 8.1.

8.2 Actions and Uses of Monoclonal Antibodies in Cancer

MAbs have been used in the treatment of cancer for the following purposes:

- Direct modulation of tumor function
- Promotion of tumor lysis by immune effector cells
- Immunization of patients against tumor antigens
- Induction of apoptosis
- Antiangiogenic effect
- Disruption of signaling
- Targeted radiolysis

There are multiple mechanisms for explaining anticancer effects of MAbs, and more than one mechanism may be involved in the action of a product. Anti-EGFR MAbs promote a slow endocytic process distinct from the rapid EGF-induced receptor internalization. Combining MAbs that engage distinct epitopes significantly accelerates receptor degradation. In addition, MAb combinations have been found to be more effective than the use of single antibodies in inhibiting HER2 signaling in vitro and tumorigenesis in animals. The use of MAbs for the therapy of cancer is one of the major advances in cancer immunology. Modulation of immune system by interplay with tumor cells through targeting of T-cell receptors (TCRs) has emerged as a powerful new therapeutic strategy for tumor therapy and to enhance cancer vaccine efficacy (Scott et al. 2012). One of the major challenges now is to combine two major immune-based treatments—MAbs and vaccines. A series of clinical trials is exploring this approach.

8.2.1 Targeted Antibody-Based Cancer Therapy

8.2.1.1 Antibody–Cytokine Fusion Proteins

Antibody–cytokine fusion proteins consist of cytokines fused to an antibody to improve antibody-targeted cancer immunotherapy. These molecules have the capacity to enhance the tumoricidal activity of the antibodies and/or activate a secondary

Table 8.1 Monoclonal antibodies for cancer approved by the FDA

Drug/year of approval	Company	Type	Target	Indication
Alemtuzumab (Campath) 2001	Millennium and Ilex Partners	Humanized MAb	CD52	B-cell chronic lymphocytic leukemia
Bevacizumab (Avastin)/2004	Genentech	Humanized MAb	VEGF	Metastatic colorectal cancer Combined with 5-FU
Cetuximab (Erbitux)/2004	ImClone Systems/Bristol-Myers Squibb	IgG1 chimeric MAb	EGFR	Metastatic colorectal cancer Combined with irinotecan
Daclizumab (Zenapax)/2002	Protein Design Labs	Chimeric MAb	IL-2R	Leukemia
Gemtuzumab (Mylotarg)/2000	Wyeth	Humanized MAb	CD33	Acute myeloid leukemia
Ibritumomab (Zevalin)/2002	Cell Therapeutics Inc.	Murine MAb/^{90Y}	CD20	Non-Hodgkin lymphoma
Ipilimumab (Yervoy)/2011	Bristol-Myers Squibb	Humanized MAb	CTLA-4	Melanoma
Ofatumumab (Arzerra)/2009	Genmab	Humanized MAb	CD20	B-cell chronic lymphocytic leukemia
Panitumumab (Vectibix) 2006	Amgen Inc.	Human XenoMouse®	EGFR	EGFR-expressing metastatic colorectal cancer
Rituximab (Rituxan)/1997	Genentech/Biogen Idec	Chimeric MAb	CD20	Non-Hodgkin lymphoma, chronic lymphocytic leukemia
Tositumomab (Bexxar)/2003	Corixa/GlaxoSmithKline	Murine MAb/^{131}I	CD20	Non-Hodgkin lymphoma, chronic lymphocytic leukemia
Trastuzumab (Herceptin)/1998	Genentech/Roche	Humanized MAb	HER-2/neu	Breast cancer, NSCLC, pancreatic cancer

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CTLA-4 cytotoxic T lymphocyte-associated antigen 4, *EGFR* epidermal growth factor receptor, *VEGF* vascular endothelial growth factor, *NSCLC* non-small-cell lung cancer

antitumor immune response. A review of studies of multiple antibody–cytokine fusion proteins, including preclinical and clinical studies focusing on IL-2, IL-12, and GM-CSF, has demonstrated significant antitumor activity as direct therapeutics or as adjuvants of cancer vaccines (Ortiz-Sanchez et al. 2008). Tumor-targeting ability of scFv(L19) in patients with cancer has been demonstrated using scintigraphic methods, and scFv(L19)-based antibody–cytokine fusion proteins are currently in clinical trials.

8.2.1.2 Antibody J591 for Targeted Delivery of Anticancer Therapy

Since the prostate-specific membrane antigen (PSMA) exists only on tumors and not other tissues, J591 armed with a drug or radiation offers a way to selectively target cancer while leaving healthy tissues unharmed, thereby resulting in very low levels of toxicity and fewer side effects for patients. A clinical trial has for the first time proven that J591 could exclusively target tumors without hitting normal tissues. Although it does not reduce tumor size, it provides a vehicle for selectively transporting drugs or a radioactive isotope to destroy the blood vessels that feed tumors, thereby cutting off the tumor's blood supply. Acceptable toxicity and excellent targeting of known sites of metastases were demonstrated in patients with multiple solid tumor types, highlighting a potential role for the anti-PSMA antibody J591 as a vascular-targeting agent (Milowsky et al. 2007). The trial involved cancer patients with a wide range of solid tumors including kidney, bladder, lung, breast, colorectal, pancreas, and melanoma. A radioactive tracer, attached to the antibody, was used to follow J591's progress throughout the body. The next step will be to arm the J591 antibody with drugs or radioactivity and then will assess tumor response. Such armed antibodies have been used in patients with prostate cancer and show significant antitumor activity has been demonstrated. Antiangiogenic agents are less effective against more advanced tumors with established blood vessels. By directly targeting tumor blood vessels, however, J591 treatments could destroy the tumor's blood supply and shrink even advanced tumors.

8.2.1.3 Anti-Thomsen–Friedenreich Antigen MAb

Thomsen–Friedenreich antigen (TF-Ag) is expressed in many cancers, including those of the breast, colon, bladder, and prostate. TF-Ag is important in adhesion and metastasis and as a potential immunotherapy target. Passive transfer of JAA-F11, an anti-TF-Ag MAb, was demonstrated to bind with a carbohydrate on the tumor cell surface that is involved in adhesion of the cell during the metastatic process and blocked metastases in murine lung vasculature in an in vivo metastatic deposit formation assay (Heimberger et al. 2009). JAA-F11 significantly extended the median survival time of animals bearing metastatic 4T1 breast tumors and caused a >50 % inhibition of lung metastasis. Not only would drugs attached to the antibody

JAA-F11 bind to the tumor cell surface to direct their cytotoxic effect, but the binding of the antibody itself would block the cell from metastasizing. Currently research is determining if JAA-F11 could increase the effectiveness of existing cancer drugs as well as studying the possibility of using the antibody as a vehicle for the targeted delivery of drugs to aid cancer diagnosis and therapy.

8.2.1.4 Bavituximab

Bavituximab (Peregrine Pharmaceuticals) is an MAb that targets phosphatidylserine (PS) and represents a new approach to the treatment of cancer. PS is a highly immunosuppressive molecule usually located inside the membrane of healthy cells but gets displaced to be exposed on the exterior of cells that line tumor blood vessels, enabling a tumor to evade immune detection. Cancer therapies increase PS exposure on the cell surface, further increasing immune suppression in the tumor environment, whereas bavituximab targets PS and blocks this immunosuppressive signal, resulting in the maturation of dendritic cells and cancer-fighting (M1) macrophages leading to the development of cytotoxic T cells for fighting solid cancers.

Bavituximab and other PS-targeting antibodies have shown anticancer activity in several preclinical studies including models of lung, pancreatic, breast, prostate, and brain cancer. Experimental cancer vaccine regimens using bavituximab have demonstrated the potential of generating protective immune responses against specific tumor challenges. In a phase I clinical trial, bavituximab was well tolerated and pharmacokinetic studies support a weekly dosing regimen (Gerber et al. 2011). MAbs directed against exposed PS have potential as a powerful adjunct to postoperative chemotherapy in preventing relapses after cancer surgery (Judy et al. 2012). Phase I and II clinical trials are in progress to investigate bavituximab in combination with chemotherapy and other molecularly targeted agents.

8.2.1.5 Combining MAbs with Anti-CD55 Antibody

Efficacy of many therapeutic products, including MAbs, may be limited by the presence of certain proteins on tumor surfaces that protect the tumor from attack by the immune system. One of these proteins is CD55, which is expressed on most tumor cells at far higher levels than on normal cells, and its function helps prevent the destruction of tumor cells. The anti-CD55 antibody (Viragen) was shown to effectively remove this protective effect and significantly enhance the activity of rituximab (Biogen Idec's Rituxan®), when both drugs were used together in a cell-based evaluation study. The anti-CD55 antibody binds to a specific target expressed on the surface of tumor cells and removes one of the tumor's most important protective mechanisms, thereby making cancer cells vulnerable to attack by the immune system or other anticancer products. Results of the study showed that the combination of the anti-CD55 antibody and rituximab led to a significant increase in the

destruction of cancer cells as compared to rituximab alone. The anti-CD55 antibody is not approved for sale in any market and human clinical trials will be required prior to seeking approval from any international regulatory agency.

8.2.1.6 MAbs Targeted to Alpha-Fetoprotein Receptor

ProtoKinetix has generated antibodies to the RECAF™ (receptor for alpha-fetoprotein), which is a biomarker for cancer. The presence of alpha-fetoprotein in the human body (other than in pregnant women) is a nonspecific indicator of the existence of cancer in the system. MAbs specific for the RECAF site do not have an affinity to healthy cells. Anti-RECAF antibodies, modified according to ProtoKinetix's Super–Antibody proprietary technology and the Peregrine Pharmaceuticals' catalytic antibody technology, improve the contrast between cancer cells and the surrounding nonmalignant tissue. Preliminary results of a study carried out by the Georges Pompidou Hospital in France, which was designed to validate the Histo-RECAF version 2.0, are positive and indicate that labeling of the malignant cells is strong with a nice delineation between malignant and normal cells. This technology has the potential for targeted cancer therapy and is being commercialized by BioCurex Inc.

8.2.1.7 MAbs Targeted to Tumor Blood Vessels

DX-2240 (Dyax Corporation) is a fully human MAb that targets the Tie-1 receptor on tumor blood vessels and has therapeutic potential in numerous oncology indications. In preclinical animal models, DX-2240 has demonstrated activity against a broad range of solid cancers. The antibody works by altering tumor vascular morphology, thereby increasing hypoxia and necrosis. In addition, DX-2240 in vivo increases the anticancer activity of other therapies such as VEGF pathway inhibitors and chemotherapeutic agents when used in combination. Sanofi-Aventis has licensed DX-2240 from Dyax for further development.

8.2.1.8 Volociximab

Volociximab is a first-in-class chimeric MAb that targets α5β1 integrin. Preclinical studies have shown the ability of volociximab to inhibit tumor neoangiogenesis by blocking the interaction between α5β1 and fibronectin. Volociximab's safety profile, pharmacokinetics, and pharmacodynamics have been established. Ongoing clinical trials are evaluating its efficacy in the treatment of different types of solid tumors as a single agent or in combination with chemotherapy. It has shown promising activity in different types of cancer (Almokadem and Belani 2012).

8.2.2 MAbs for Immune Activation

The mechanism by which low doses of MAbs activate immune responses to tumor-specific antigens is, in part, analogous to the mechanism of a classic technique in experimental immunology used to produce antibodies against molecules that usually do not elicit an immune response. In this classic technique, the molecule of interest is attached to foreign antibody that is highly immunogenic by itself. In the process of attacking the foreign antibody, the body is also "tricked" into mounting an immunological reaction against the targeted molecule (tumor-associated antigen [TAA]) now attached to the protein.

MAbs in development by AltaRex Corporation serve as large highly immunogenic proteins that bind to tumor-specific antigens. Very low doses of MAbs, administered intravenously, effectively induce this potentially therapeutic immune response. The lead product, OvaRex® MAb, has shown promise for the treatment of ovarian cancer patients in both remission and recurrent stages of the disease. OvaRex® MAb is documented to induce cellular and humoral immune responses against the tumor-specific antigen CA-125, which is the most thoroughly studied serum marker for ovarian cancer. There is a correlation between the extent of the immunogenic response against CA-125 and progression-free and/or survival time of patients. The antibodies generated in response to the administration of OvaRex MAb are directed against multiple epitopes of the CA-125 molecule, indicating a highly effective immune induction in response to the product.

8.2.3 Delivery of Cancer Therapy with MAbs

MAbs can be used for the delivery of cytotoxin payloads such as radionucleotides, toxins, and chemotherapeutic agents to the tumors as shown in Table 8.2.

8.2.4 Antibody-Directed Enzyme Prodrug Therapy

Antibody-directed enzyme prodrug therapy (ADEPT) involves both an antibody–enzyme conjugate and a low-toxicity prodrug. First the antibody–enzyme conjugate is delivered to the target cells that express tumor antigens on their surface. The unbound antibody is allowed to clear before the prodrug is administered. The enzyme part of the conjugate then cleaves the prodrug to unleash the active form of the drug at the tumor site. Not all tumor cells need to be targeted because activated drug accumulates and diffuses at the tumor site, killing nearby cells.

Previous clinical trials have shown evidence of tumor response, but the activated drug had a long half-life, which resulted in dose-limiting myelosuppression.

Table 8.2 Anticancer agents linked to monoclonal antibodies

Drugs
Anthracyclines: doxorubicin, daunorubicin, idarubicin, nemorubicin
Antimetabolites: aminopterin, methotrexate, fluorouracil, floxuridine, cytarabine
Alkylating agents: melphalan, chlorambucil, mitomycin, cisplatin, trenimon
Antimitotic drugs: vinca alkaloids, podophyllotoxin, colchicine
DNA intercalations: ethidium dimer neocarzinostatin, tyrosine kinase inhibitor genistein
Toxins
Pseudomonas exotoxin
Ricin
Saporin
Diphtheria toxin
Isotopes
^{131}Iodine
^{99}Technetium
^{90}Yttrium
Enzymes
β-Lactamase
Alkaline phosphatase
Antibody-directed enzyme prodrug therapy (ADEPT)
Carboxypeptidase
Cytidine deaminase
Glucose oxidase
Cytokines
TNF-α
Interferon
Interleukin-2
Sensitizers
Radiosensitizers
Photosensitizers
Miscellaneous agents
MRI contrast agents

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Also, the targeting system, although giving high tumor-to-blood ratios of antibody–enzyme conjugate (10,000:1), required the administration of a clearing antibody in addition to the antibody–enzyme conjugate. Because ADEPT therapy uses an enzyme carboxypeptidase G2 (CP) not naturally found in humans, a patient's immune system can recognize and reject the enzyme before it can do its job. This has been addressed by identification and disguising clinically important immunogenic sites on recombinant fusion proteins to enable them to repeatedly elude the immune system. The advance could allow patients to safely receive multiple courses of ADEPT to treat their cancer.

8.2.5 Chemically Programmed Antibodies

Studies at the interface of chemistry and biology have enabled the development of an immunotherapeutic approach called chemically programmed antibodies (cpAbs), which combines the merits of traditional small-molecule drug design with immunotherapy and breaks the “one antibody–one target axiom” of immunochemistry. Small-cell-targeting molecules used with a nontargeting catalytic aldolase MAb 38C2 create a novel compound for retargeting it to breast cancer cell lines expressing integrin αvβ3. This approach enables the effective assembly of cpAbs in vitro as well as in vivo and intracellular delivery of cpAbs into cells. A substantial enhancement of the therapeutic effect over the peptidomimetic itself has been demonstrated in animal models of breast cancer metastases.

This technology possesses potential for the diagnosis as well as treatment of disease. An imaging agent could be attached to the small molecule, enabling a physician to monitor the localization of a drug before arming the agent with the antibody molecule. Whereas treatment with MAbs requires a different antibody for each specific target, cpAbs approach enables different small-molecule-targeting agents, called programming agents or adapters, to selectively direct the same MAb to different sites for different uses so that only a single antibody is required for multiple tasks. Among the potential therapeutic advantages is a dramatically increased circulatory half-life of the compound, which could give patients greater exposure to the benefits of any treatment. The new hybrid compound remained in circulation for a week as compared to a small-molecule drug that is cleared in a matter of minutes. cpAB approach has been used in a melanoma model, dramatically enhancing the effectiveness of a small-molecule drug (Popkov et al. 2006).

8.2.6 Combination of Diagnostics with Therapeutics Based on MAbs

Two tests—Poteligeo Test IHC (immunohistochemistry) and Poteligeo Test FCM (Kyowa Medex)—were approved in Japan in March 2012 as companion diagnostics for mogamulizumab (Kyowa Hakko Kirin’s Poteligeo) injection, a therapeutic MAb for the treatment of adult T-cell leukemia (ATL). Poteligeo binds to CCR4, which is expressed on the surface of ATL cells which are killed by MAb-dependent cell-mediated cytotoxicity. The companion diagnostic tests detect the presence of CCR4 expressed by ATL cells before treatment with Poteligeo to enable the identification of patients who would benefit from the drug. Poteligeo Test IHC is for use on tissue samples, such as lymph nodes, whereas Poteligeo Test FCM uses flow cytometry to analyze blood samples from patients.

8.2.7 Radiolabeled Antibodies for Detection and Targeted Therapy of Cancer

Radiolabeled MAbs are widely used in the detection and treatment of cancer. There are several preparations and a few examples will be described here.

8.2.7.1 Ibritumomab Tiuxetan

Ibritumomab tiuxetan (Zevalin), the first MAb conjugated with a radionucleotide to be approved, is a radioimmunotherapy marketed for certain types of B-cell non-Hodgkin lymphoma (NHL) in the USA. It is a chimeric MAb of murine origin, but over 95 % of it is humanized. It binds to the same target as Rituxan but complements Rituxan by killing NHL cells through a different mechanism. Binding of Rituxan to CD20 on the tumor cell surface recruits the immune system to destroy the cell. It destroys tumor with the radionuclide yttrium-90 that is tightly bound to the antibody by means of a covalently linked chelator. Yttrium-90 releases a beta particle that can penetrate surrounding tumor cells over a 5-mm radius, leaving cellular free radicals followed by cell death, in its wake. As a radiolabeled antibody, murine origin is an advantage. A shorter half-life decreases yttrium-90 circulation time, and risk of provoking human antibodies is low, possibly due to its dose being orders of magnitude smaller than that of Rituxan. The vastly lower required dose reflects the enhancement of potency by the addition of the radionuclide.

8.2.7.2 huHMFG1

huHMFG1 (formerly Therex) is a humanized version of the mouse MAb HMFG1 (human milk fat globule-1). It binds to MUC1, a cell membrane protein present in a variety of tumors of epithelial origin, including breast, ovarian, pancreatic, gastric, and colon cancers. R1550 acts as a “marker flag,” attaching itself to tumor cells and helping components of the immune system, including natural killer cells, to find and destroy them. Humanization is intended to make the antibody less immunogenic (i.e., to make the antibody less likely to be recognized as foreign material by the patient’s immune system) and thus increase its suitability for repeat intravenous administration. It has undergone a phase I trial in metastatic breast cancer and pharmacokinetics was characterized (Royer et al. 2010).

8.2.7.3 KW-2871

KW-2871 (Kyowa Hakko Kirin Ltd.). KW-2871, a chimeric antibody made by POTELLIGENT™ technology, is under development for stage IV malignant melanoma and is in phase I/IIa clinical trials in the USA. POTELLIGENT technology boosts the efficacy of antibodies. When an antibody had a reduced amount of fucose

in its sugar chains, it exhibits much higher antibody-dependent cellular cytotoxicity (ADCC) activity as compared to a highly fucosylated conventional antibody. The mechanism behind the enhanced ADCC of a low-/no-fucose antibody is its increased affinity to FcγRIIIa (CD16), the major Fc receptor for ADCC in humans. Knockout of a gene called FUT8 that is responsible for the fucose addition to sugar chains created a new production method for fucose-free antibodies in Chinese Hamster Ovary (CHO) cells. Finally the FUT8-knockout CHO cells (POTELLIGENT® Cells) were created, which are shown to produce fucose-free, thereby ADCC-enhanced antibodies (POTELLIGENT® MAbs), with unchanged basic properties (stability, growth rate, productivity, and scalability) compared to parental CHO cells.

8.2.7.4 Ferritarg

Ferritarg (MAT Biopharma), a rabbit polyclonal antibody that is coupled with either yttrium-90 or indium-111 and is directed against acidic ferritin, is used for radioimmunotherapy. Ferritin is produced by most cells in the human body and the protein's function is to bind and store iron. Elevated levels of different variants of ferritin (isoferritins) have been measured on the surface of various types of cancer cells including those from cancers of the liver, lung, pancreas, head and neck, kidney, ovaries, intestines, stomach, and breast, as well as NHL and Hodgkin lymphoma. This upregulation of ferritin in cancer cells differentiates these cells from normal cells and ferritin is thus a cancer cell antigen. Ferritarg is being developed initially for the treatment of refractory Hodgkin disease. Ferritarg has been granted orphan drug status in the EU and the USA for the treatment of Hodgkin disease. It is phase I/II clinical trials.

8.2.7.5 Cotara

Cotara (Peregrine Pharmaceuticals) is a TNF-targeting chimeric MAb, labeled with ^{131}I, which is in phase III clinical trials for glioblastoma multiforme. Cotara links a radioactive isotope to a targeted MAb designed to bind to the DNA histone complex that is exposed by dead and dying cells found at the center of solid tumors. Cotara's targeting mechanism enables it to bind to the dying tumor cells, delivering its radioactive payload to the adjacent living tumor cells and essentially destroying the tumor from the inside out, with minimal radiation exposure to healthy tissue. Cotara is delivered in a single dose using convection-enhanced delivery, a method that targets the specific tumor site in the brain.

8.2.7.6 Clivatuzumab

Clivatuzumab (Immunomedics) is a humanized MAb targeting a mucin antigen expressed in most pancreatic cancers, but not pancreatitis, normal pancreas, or most

other normal tissues. The yttrium-90-labeled MAb has orphan drug status in both the USA and the EU and fast track status in the USA for the treatment of pancreatic cancer. Preclinical studies in mice with human pancreatic cancer xenografts given the murine version of yttrium-90 clivatuzumab tetraxetan demonstrated favorable tumor responses, which could be further improved when given in combination with gemcitabine. A prior phase I single dose-escalation study of yttrium-90 clivatuzumab tetraxetan in treatment-relapsed pancreatic cancer patients has also produced encouraging results, with evidence of objective responses. A phase III clinical trial of yttrium-90 clivatuzumab in combination with gemcitabine is ongoing for the treatment of patients with newly diagnosed, inoperable, untreated stage III or stage IV cancer of the pancreas.

8.2.8 Methods for Increasing MAb Selectivity for Tumor Tissue

MAb selectivity for tumor tissue can be increased. Some of the improvements are as follows:

Bispecific Antibodies. Using either hybridoma fusion, chemical methods, or genetic engineering technology, antibodies with dual specificity can be constructed. These so-called bispecific antibodies have been used to redirect the cytolytic activity of a variety of immune effector cells such as cytotoxic T lymphocytes (CTLs), natural killer cells, neutrophils, and monocytes/macrophages to tumor cells.

Immunomedics has designed a bispecific fusion protein by linking portions of genes encoding two distinct antibodies to yield a single protein molecule with two binding sites: one to the tumor and the other to a small molecule carrying a therapeutic agent. The fusion protein is half the size of a typical antibody and is substantially humanized by molecular engineering. In the first step, the bispecific antibody fusion protein is injected intravenously and subsequently binds to cancer cells, while unbound material is cleared rapidly from the body. In the second step, a small drug carrier is injected and shows specific uptake into the tumors that have been bound by the fusion protein. As early as 4 h after injection of the small-molecule drug carrier, researchers measured a 13:1 ratio of uptake of the therapeutic drug in the tumor as compared with the blood. This is an eightfold higher tumor selectivity ratio than that achieved with the directly antibody-bound drug. In experiments with chemically conjugated fusion proteins, drug targeting resulted in almost 100 times more uptake in the tumor than in normal tissues. It is in phase II clinical trials with a carcinoembryonic antigen (CEA) bispecific antibody in patients with CEA-expressing tumors, such as colorectal and medullary thyroid cancers.

Currently, systemic application of bispecific antibodies is suitable only for adjuvant treatment of minimal residual disease because of poor tumor cell accessibility. As an alternative, attacking the tumor's blood supply by delivering coagulation factors or toxins or by bispecific antibody-directed immunotherapies holds great promise for anticancer therapy.

Trifunctional Antibodies. These antibodies are called trifunctional because they bind to cancer cells and also to two different cells of the immune system: T cells and macrophages. Through the creation of this cell complex, the trifunctional antibodies initiate an especially efficient eradication of tumor cells. The goal is to eliminate those tumor cells that may still be present in the body, e.g., following surgical resection of a tumor—to prevent relapse or the development of metastases. The trifunctional antibodies were developed by TRION Pharma, a partner of Fresenius Biotech, which successfully completed a phase I/II study of the treatment of ascites in ovarian cancer using the trifunctional antibody Removab®. The antibody was shown to be well tolerated and demonstrated the first significant indications of efficacy. The following additional studies with Removab® and the other trifunctional antibody Rexomun™ have been done:

- Two phase I studies have investigated the use of Rexomun™ in breast cancer.
- Phase II/III studies have investigated Removab® in NSCLC, malignant pleural effusion, stomach cancer, pancreatic cancer, and ovarian cancer.

Immunotoxins. MAbs can also be coupled with cellular toxins. One of the most potent ones is *Pseudomonas* exotoxin. Proxinium (Viventia Biotech) is a protein engineered from the fusion of a truncated form of *Pseudomonas* exotoxin A to the humanized scFv, which is specific for the epithelial cell adhesion molecule (Ep-CAM). It is in phase II clinical trials for head and neck cancer. SS1P (Enzon Pharmaceuticals) is a recombinant immunotoxin consisting of anti-mesothelin MAb fragment and *Pseudomonas* exotoxin. It is in phase II clinical trials for pancreatic and ovarian cancers.

Local Injection of MAbs or MAb Complexes. Such injections can be made directly into the tumors. This method is useful for chemotherapy of breast cancer nodes in the adjoining lymph system.

Immunoliposomes (Antibody-Coupled Liposomes). Attempts have been made at targeting MAbs to the tumor site by binding them to liposomes. Some of the problems regarding immunoliposome preparation and application such as antibody coupling and immunoliposome stability and pharmacokinetics have been overcome during the last decade. Some of the challenges that still need further work are:

- Difficulty of maintaining the biological activity of therapeutic proteins when they are attached to polyethylene glycol (PEG)-liposomes
- Difficulty of releasing the drug from the immunoliposome complex
- Limited capacity of the immunoliposomes to carry drugs

Fab type C showed low reticuloendothelial system (RES) uptake and a long circulation time and enhanced accumulation of the liposomes in the solid tumor. The small Fab type C (PEG-immunoliposomes) predominantly passes through the leaky tumor endothelium by passive convective transport and provides an important insight into the potential of type C liposomes for target-specific drug delivery.

Immunoliposomes that target EGFR and/or its truncated variant EGFRvIII can be constructed to provide efficient intracellular drug delivery in tumor cells

overexpressing these receptors. MAb fragments can be covalently linked to liposomes containing various reporters or drugs such as doxorubicin, vinorelbine, or methotrexate. In each case, the immunoliposome agent is significantly more cytotoxic than the corresponding nontargeted liposomal drug in target cells, whereas it is equivalent in cells lacking EGFR/EGFRvIII overexpression. Anti-HER2 immunoliposomes containing HERMES Bioscience's proprietary antibody fragment F5 and the chemotherapy drug doxorubicin are in development toward clinical trials.

Combined Use of MAbs and Cytokines. The potent antitumor activity of certain cytokines is often achieved at the expense of unacceptable toxicity. One avenue to improve the therapeutic index of cytokines in cancer therapy consists of fusing them to MAbs capable of a selective localization at the tumor site. Fusion proteins of IL-12 and TNF-α with L19, an antibody fragment specific to the extra domain B of fibronectin which has been shown to target tumors in animal models and in patients with cancer. These fusions display a potent antitumor activity in several immunocompetent murine models of cancer but do not lead to complete remissions of established aggressive tumors. They have further evaluated the tumor-targeting properties and the anticancer activities of combinations of the two antibody–cytokine fusion proteins, as well as of a triple fusion protein between IL-12, L19, and TNF-α. Although all fusion proteins were active in vitro, the triple fusion protein failed to localize to tumors in vivo and to show significant therapeutic effects. By contrast, the combination of IL-12–L19 and L19–TNF-α displayed potent synergistic anticancer activity and led to the eradication of F9 teratocarcinomas grafted in immunocompetent mice. When cured mice were rechallenged with tumor cells, a delayed onset of tumor growth was observed, indicating the induction of a partial antitumor vaccination effect. The combined administration of the two fusion proteins showed only a modest increase in toxicity, compared with treatments performed with the individual fusion proteins. These results show that the targeted delivery of cytokines to the tumor environment strongly potentiates their antitumor activity and that the combination treatment with IL-12–L19 and L19–TNF-α appears to be synergistic in vivo.

huHMFG1–huDNase I. It is known that HMFG1 is internalized. In addition, DNase has been shown to be highly toxic when injected directly into isolated tumor cells. It kills the cells by inducing apoptosis, which causes the cells to break down into small particles that can be cleared by natural scavenger cells. This may avoid the potentially life-threatening inflammatory responses experienced by some cancer patients treated with conventional therapies. In vitro experiments have confirmed that, while neither the antibody nor the enzyme alone is toxic, the combination of a humanized MAb HMFG1 with the enzyme DNase demonstrates rapid cell killing. The gene for the DNase-based drug has been stably inserted into a human cell line, an important step toward the development of a reliable manufacturing process. In addition, it has been shown that cells containing this gene produce a substance that targets and kills tumor cells.

MAbs That Selectively Target Cancer. Micromet's fully human MAb MT201 (adecatumumab) is directed against the Ep-CAM. The product is currently being

tested in two multicenter phase II clinical trials for the treatment of prostate and metastatic breast cancer. Ep-CAM, the target antigen for MT201, is overexpressed with high frequency on most human carcinomas, suggesting that it may have therapeutic potential in the treatment of a broad range of cancers, including prostate, breast, colon, lung, stomach, pancreatic, head and neck, and ovarian cancer. MT201 has been specifically designed to selectively eliminate tumor cells while leaving healthy tissues largely unharmed. Phase I data have demonstrated an excellent safety profile of MT201, and no MT201-neutralizing antibodies have been observed so far in man. An Investigational New Drug (IND) for the product has been cleared by the FDA for the initiation of phase II studies in the USA.

MAb 806 (Life Science Pharmaceuticals) specifically targets EGFR on a wide range of tumor types but has no uptake by normal tissues. The 806 antigen is not exposed on inactive wild-type EGFR, but is exposed on a transitional form of the EGFR. The epitope studies are supported by IHC demonstrating that the 806 antibody binds to a broad range of epithelial cancers and to gliomas, but not to normal human tissues. Other preclinical data suggest that MAb 806 would not have the side effects observed with other EGFR-targeting MAbs. Results of a clinical trial confirmed the target specificity and safety of MAb 806 in human patients with a variety of cancers including squamous cell carcinomas of the lung, head and neck, and skin; colorectal cancer; mesothelioma; and glioma (Scott et al. 2006).

8.2.9 Advantages and Limitations of MAbs for Cancer Therapy

MAbs offer the following advantages for cancer therapy:

- High specificity for tumor antigens
- Long half-life which enables reduction of dose frequency
- Low cross-reactivity with normal cells
- Possibility of manufacture in large quantity and with a high degree of purity
- Flexibility of design: customization with respect to immunoglobulin binding sites
- Shorter development time compared to traditional small-molecule drugs

MAbs are likely to enhance, rather than replace, current cancer therapies by targeted destruction of cancer cells and possible recruitment of the body's immune system. Although MAbs have been proposed as vehicles to target cancer cells specifically, several theoretical factors for their safety/efficacy have been a major concern. MAbs have the following limitations:

- Cellular targets are restricted to surface antigens as their large size prevents direct cell penetration and important intracellular protein targets remain inaccessible. Since cancer cells express only 10–15 % of proteins on cell surface, the remaining 85–90 % cannot be targeted.

- Slow elimination from the blood, poor vascular permeability, and low tumor uptake.
- Tumor selectiveness rather than tumor specificity, i.e., they can bind to normal cells that have the same tumor receptors as tumors.
- Antigenic heterogeneity.
- Induction of human anti-mouse antibody responses.

Alternatives to MAbs to overcome some of the limitations include single-chain antibody-binding (SCA) protein technology, monoclonal TCR technology, and antibody–drug conjugates (ADCs). These are described in the following sections.

8.3 Single-Chain Antibody-Binding Protein Technology

SCA proteins, like MAbs, deliver therapeutic proteins to targeted disease sites. The advantages over MAbs are:

- SCA proteins are easier to produce and do not need to go through the humanization process.
- SCAs penetrate the tumor much better because their molecular weight is only a fraction of that of the usual antibodies.
- Immunogenicity is reduced because protein that is not required for antigen binding is not included.
- Flexibility to tailor half-life via PEG technology.
- More cost-effective scale-up for manufacturing when compared with MAbs.
- Better delivery opportunities offering potential for non-parenteral delivery.

Enzon is developing SCA technology for the delivery of cancer therapeutics. Using an ^{123}iodine-labeled SCA selected from a combinatorial library, clinical evidence of efficient tumor targeting in patients with CEA-producing cancer has been demonstrated.

8.4 Monoclonal T-Cell Receptor Technology

T cells are a powerful defense against cancer cells. Every peptide antigen has a corresponding T cell, which can bind to its target via the TCR on its surface. T cell, thus activated, destroys the cancer cell. TCRs, isolated in a soluble form and engineered like antibodies, have the potential to become specific and sensitive tools for targeting cancer. They can overcome some of the limitations of conventional monoclonal antibodies. TCRs differ from MAbs in antigen recognition. MAbs recognize and target TAAs expressed as membrane-bound proteins on the surface of tumor cells. Some of these are tumor type specific, whereas others are expressed in a wide variety of tumors. Cancer cells may also present tumor-associated peptide antigens (TAPAs), some of which are derived from intracellular proteins and are tumor-specific and useful targets for therapeutic intervention. TAPAs are not recognized by antibodies but by TCRs. Monoclonal TCRs (mTCRs) thus represent a new

approach to the targeted treatment of cancer. While retaining the benefits of MAbs, mTCRs provide additional benefits such as enhanced ability to target the breakdown products of intracellular proteins and to deliver cytotoxic agents to cancer cells.

There are, however, technical problems of using TCRs. Unlike antibodies, TCRs are not expressed in a soluble form, but are anchored to the T-cell surface by an insoluble transmembrane domain. Characterization and development of TCRs have been hampered by the lack of suitable methods for producing them as soluble and stable proteins. mTCR technology (Immunocore) enables the production of fully human, soluble TCRs and links them to an antibody fragment, anti-CD3, which can activate the immune system to kill the targeted cancer cells. ImmTACs (Immune mobilizing mTCR Against Cancer), which are bispecific biologics comprising a soluble, high-affinity TCR fused to a cluster of differentiation 3 (CD3)-specific single-chain antibody fragment (scFv), effectively redirect T cells to kill cancer cells expressing extremely low surface epitope densities (Liddy et al. 2012). ImmTACs potently suppress tumor growth in vivo and overcome immune tolerance to cancer. Lead product, IMCgp100, is currently in a phase I/II dose-finding clinical study in patients with late-stage malignant melanoma.

8.5 Antibody–Drug Conjugates

ADCs combine the high selectivity of MAbs with potency of small molecules to increase the anticancer effect. MAbs directed to TAAs or antigens differentially expressed on the tumor vasculature have been covalently linked to drugs that have different mechanisms of action and various levels of potency. The use of ADCs to selectively deliver drugs to tumors has the potential to both improve antitumor efficacy and reduce the systemic toxicity of therapy. Several ADCs, particularly those that incorporate internalizing antibodies and tumor-selective linkers, have demonstrated impressive activity in preclinical models. Gemtuzumab ozogamicin (Mylotarg, Pfizer), a calicheamicin conjugate that targets CD33, was approved by the FDA for the treatment of acute myelogenous leukemia but was withdrawn from the market later on.

ADCs, however, are not just a sum of their individual parts and several challenges need to be addressed. The target selection, the interaction of ADC with tumor and off-tumor targets, and the internalization of ADCs are critical for the effective maturation of ADC technology. Ongoing developments in attachment sites and linker chemistry can provide fine-tuning of drug loading, elements of ADC pharmacokinetics, and off-target ADC toxicity (Adair et al. 2012).

Sutro Biopharma is using its cell-free protein synthesis technology platform to design and develop new ADCs and bispecific antibodies for targeted cancer therapies of Celgene. Sutro's biochemical synthesis technology enables rapid and systematic exploration of many protein-drug variants to identify drug candidates. The new treatments are aimed at significantly extending the clinical impact of current oncology therapeutic approaches beyond that of current cell-based expression technologies.

Spirogen's ADC technology delivers extremely potent anticancer agents to cancer cells by attaching them to antibodies. Spirogen has developed highly potent warheads based on its proprietary pyrrolobenzodiazepines (PBDs) warheads joined to antibodies by linkers, which are stable in the bloodstream but release the PBD warhead once it is safely inside the targeted cancer cells. The naturally occurring PBDs, isolated from various *Streptomyces* species, bind covalently and sequence selectively to purine–guanine–purine motifs in the minor groove of DNA. The DNA-binding activity of the molecules interferes with DNA processes including transcription and replication, allowing them to act as antitumor and antibiotic agents. The PBD dimer SG2000 is currently undergoing phase II clinical trials for the treatment of cisplatin-resistant ovarian cancer.

Currently ~60 ADCs are in development for oncology including ~20 in clinical trials, most of which are tubulin inhibitor-based immunoconjugates (Sapra et al. 2011). Only two, Kadcyla and Adcetris, have been approved. Selected 15 clinical trials of ADC are shown in Table 8.3.

ADCs are predicted to become an important class of cancer therapeutics as evidenced by the promising objective response rates when administered to chemo-refractory cancer patients. Further improvements are being made to ADC design, and a third generation of agents is already emerging. Mersana Therapeutics is focusing on developing ADCs using its Fleximer polymer backbone and customized linkers to optimally link MAb and drug.

8.5.1 Ado-Trastuzumab Emtansine

Ado-trastuzumab emtansine (Roche/Genentech's Kadcyla) was the first FDA-approved ADC for treating HER2-positive metastatic breast cancer, an aggressive form of the disease. Ado-trastuzumab emtansine is made up of the antibody, trastuzumab, and the chemotherapeutic, mertansine (DM1), joined together using a stable linker. It combines the mechanisms of action of both trastuzumab and DM1. Genentech has studied ADC science for more than a decade and has several ADCs in clinical trials for different types of cancer as well as >25 candidates in pipeline. Results of a randomized clinical trial, EMILIA study, showed that T-DM1 significantly prolonged progression-free and overall survival (~6 m) with less toxicity than lapatinib plus capecitabine in patients with HER2-positive advanced breast cancer previously treated with trastuzumab and a taxane (Verma et al. 2012).

8.5.2 Brentuximab Vedotin

Brentuximab vedotin (Seattle Genetics' Adcetris) consists of an anti-CD30 antibody, a cell membrane protein of the TNF family, conjugated to the antimitotic agent monomethyl auristatin E (Bradley et al. 2013). It was approved by the FDA for the treatment of Hodgkin lymphoma (HL) and systemic anaplastic large-cell

Table 8.3 Antibody–drug conjugates in clinical trials for cancer

Product	Target antigen/drug	Company	Indication	Phase
AGS-16M8F	AGS-16/auristatin	Agensys/Astellas Pharma	Renal cell cancer	Phase I
AGS-5ME	AGS-5/auristatin	Agensys/Astellas Pharma	Prostate, pancreatic, gastric cancers	Phase I
BAY 79-4620 (CAIX/ADC)	MN carbonic anhydrase IX (CAIX)/auristatin	Bayer Pharma/MorphoSys	Solid cancers expressing CAIX	Phase I
BAY 94-9343 (mesothelin-ADC)	Mesothelin/maytansinoid TAP	Bayer Pharma/ImmunoGen Inc.	Mesothelioma	Phase I orphan
BIIB015	Cripto/maytansinoid	Biogen/ImmunoGen Inc.	Cripto-positive solid tumors	Phase I
BT-062	CD138/maytansinoid	Biotest/ImmunoGen Inc.	Multiple myeloma	Phase I
Glembatumumab vedotin (CDX-011)	Glycoprotein NMB (GPNMB)/auristatin	Celldex Therapeutics	Breast cancer expressing GPNMB	Phase II
IMGN-388	Integrin αv/maytansinoid	ImmunoGen Inc.	Solid tumors	Phase I
Inotuzumab ozogamicin (CMC-544)	CD22/calicheamicin	Pfizer	Non-Hodgkin lymphoma	Phase III
Lorvotuzumab mertansine (IMGN-901)	CD56/maytansinoid	ImmunoGen Inc.	Multiple myeloma, Merkel cell carcinoma, ovarian cancer	Phase I/II
MDX-1203	CD70/duocarmycin	Medarex/Bristol-Myers Squibb	Renal cell cancer	Phase I
PSMA ADC	PSMA/auristatin	Progenics Pharmaceuticals Inc.	Prostate cancer	Phase I
SAR3419	CD19/maytansinoid	Sanofi-Aventis	Non-Hodgkin lymphoma	Phase II
SG2000	Antibodies/pyrrolobenzodiazepines	Spirogen Ltd.	Cisplatin-resistant ovarian cancer	Phase II
SGN-75	CD70/auristatin	Seattle Genetics	Renal cell cancer, non-Hodgkin lymphoma	Phase I

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lymphoma (ALCL) in 2011. Approval was based on two single-arm multicenter clinical trials of patients with CD30-positive HL after failure of autologous stem cell transplant and patients with CD30-positive systemic ALCL who had previously received chemotherapy. The objective response rates were 73 % and 86 %, respectively, while the complete remission rates were 32 % and 57 %, respectively, and the partial remission rates were 40 % and 29 %, respectively. Adcetris is also in development for a range of other CD30-expressing lymphoma and non-lymphoma malignancies, both as monotherapy and in combination with chemotherapy.

8.6 Current Status and Future Trends in MAb-Based Anticancer Drugs

An analysis of the current commercial clinical pipeline of MAb candidates for cancer revealed trends toward the development of a variety of noncanonical MAbs, including ADCs, bispecific antibodies, engineered antibodies, and antibody fragments and/or domains (Reichert and Dhimolea 2012). The authors found substantial diversity in the antibody sequence source, isotype, carbohydrate residues, targets, and mechanisms of action. Although well-validated targets, such as EGFR and CD20, continue to provide opportunities for companies, there were notable trends toward targeting less well-validated antigens and exploration of innovative mechanisms of action such as the generation of anticancer immune responses or recruitment of cytotoxic T cells.

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Chapter 9
Nanooncology

9.1 Introduction

Nanotechnology (Greek word nano means dwarf) is the creation and utilization of materials, devices, and systems through the control of matter on the nanometer-length scale, i.e., at the level of atoms, molecules, and supramolecular structures. Nanotechnology, as defined by the National Nanotechnology Initiative (http://www.nano.gov/), is the understanding and control of matter at dimensions of roughly 1–100 nm, where unique phenomena enable novel applications. Encompassing nanoscale science, engineering, and technology, nanotechnology involves imaging, measuring, modeling, and manipulating matter at this length scale. It is the popular term for the construction and utilization of functional structures with at least one characteristic dimension measured in nanometers—a nanometer is one billionth of a meter (10^{-9} m).

Nanomedicine is defined as the application of nanobiotechnology to medicine. Its broad scope covers the use of nanoparticles and nanodevices in healthcare for diagnosis as well as therapeutics (Jain 2012). Application of nanotechnology in cancer can be termed nanooncology and includes both diagnostics and therapeutics (Jain 2008). Various applications in diagnosis and drug delivery for cancer are discussed in this chapter. Several nanotechnology-based products are already approved for the treatment of cancer, e.g., Doxil (a liposome preparation of doxorubicin) and Abraxane (paclitaxel in nanoparticle formulation). Over 150 drugs in development for cancer are based on nanotechnology. Some of the nanotechnologies and their applications in developing cancer therapies are described in this section. The most important factor in the fight against cancer, besides prevention, is early detection.

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_9,
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9.2 Nanobiotechnology for Detection of Cancer

Nanobiotechnology offers a novel set of tools for detection of cancer. It will contribute to early detection of cancer as follows:

- It can complement existing technologies and make significant contributions to cancer detection, prevention, diagnosis, and treatment.
- It would be extremely useful in the area of biomarker research and provide additional sensitivity in assays with relatively small sample volumes.
- Examples of applications of nanobiotechnology in cancer diagnostics include quantum dots and use of nanoparticles for tumor imaging.

9.2.1 Dendrimers for Sensing Cancer Cell Apoptosis

Poly(amidoamine) (PAMAM) dendrimers have been used as a platform for the targeted delivery of chemotherapeutic drugs in cancer. A PAMAM nanodevice can be used to monitor the rate and extent of cell killing or apoptosis caused by the delivered chemotherapeutic drug, which is important for predicting clinical efficacy (Myc et al. 2007). Whereas other approaches to detect apoptosis rely on the human protein annexin V, which binds to a hidden cell membrane component revealed in the initial stages of apoptosis, this method detects caspase-3, an enzyme activated early in the apoptosis process. This enzyme cleaves the bond between two specific amino acids and this specificity has been exploited to design fluorescence resonance energy transfer (FRET)-based assays for caspase-3. The fluorescence appears only when caspase-3 breaks a valine–aspartic acid bond in a specially designed substrate for this enzyme. To create a tumor-specific apoptosis detector, folic acid and the caspase-3 substrate were attached to a PAMAM dendrimer. Folic acid acts as a tumor-targeting agent, binding to folic acid that many types of tumor cells produce in abundance. Apoptotic tumor cells bearing this folic acid receptor take up the dendrimer and fluoresce brightly. In contrast, apoptotic cells lacking the folic acid receptor do not fluoresce. An optical fiber device, capable of detecting FRET emissions in tumors, has been used to quantify apoptosis in live mice with tumors bearing the folic acid receptor.

9.2.2 Detection of Circulating Cancer Cells

A method has been described for magnetically capturing circulating tumor cells (CTCs) in the bloodstream of mice followed by rapid photoacoustic detection (Galanzha et al. 2009). Magnetic nanoparticles, which were functionalized to target a receptor commonly found in breast cancer cells, bound and captured CTCs under a magnet. To improve detection sensitivity and specificity, gold-plated carbon nanotubes conjugated with folic acid were used as a second contrast agent for photoacoustic imaging. By integrating in vivo multiplex targeting, magnetic enrichment, signal amplification, and multicolor recognition, this approach enables CTCs to be

concentrated from a large volume of blood in the vessels of tumor-bearing mice and has potential applications for the early diagnosis of cancer and the prevention of metastasis in humans.

A nano-Velcro technology, engineered into a 2.5×5 cm microfluidic chip, is a second-generation CTC-capture technology, which is capable of highly efficient enrichment of rare CTCs captured in blood samples collected from prostate cancer patients (Wang et al. 2011a, b). It is based on the research team's earlier development of "fly-paper" technology that involves a nanopillar-covered silicon chip whose stickiness resulted from the interaction between the nanopillars and nanostructures on CTCs known as microvilli, creating an effect much like the top and bottom of Velcro. The new device adds an overlaid microfluidic channel to create a fluid flow path that increases mixing. In addition to the Velcro-like effect from the nanopillars, the mixing produced by the microfluidic channel's architecture causes the CTCs to have greater contact with the nanopillar-covered floor, further enhancing the device's efficiency. The device features high flow of the blood samples, which travel at increased speed, bouncing up and down inside the channel, get slammed against the surface, and get caught.

An affordable, nanoscale assay has been developed to quantify cancer cells that break off from tumors and circulate through the blood, which could improve cancer diagnosis and help understand how the disease spreads (Hou et al. 2013). This is a further development of the nanoscale Velcro-like material that can capture CTCs. Simply capturing the cancer cell is not enough; it also needs to be analyzed against a panel of cancer biomarkers. The new method can release these cells, leaving them intact for further analysis such as genome sequencing. The technology is also cheaper, costing <$50 to manufacture, whereas comparable assays cost ~$1,000 per run. The device consisted of an array of silicon nanowires that are coated with antibodies, which bind to a protein that lines the outer membranes of some cancer cell types called EpCAM (Wang et al. 2009). This first-generation assay captured the targeted cells but released only about half of them, and of those, only ~10 % of were viable, leaving the rest damaged. To improve on this, cell release was boosted by adding a temperature-sensitive polymer to the silicon nanowires. At 37 °C, the anti-EpCAM polymers grab tumor cells and at 4 °C they release them. As a result, ~90 % of the released cells are undamaged. Similar to the first-generation assay, the new device only separates them with 40–70 % efficiency, but there is potential to boost efficiency further by adding a microfluidic component. The assay is being validated using patient samples and a clinical trial is planned. Purity of the cell population is being improved by isolating and running samples through two assays in succession, which takes ~1 h.

9.2.3 Differentiation Between Normal and Cancer Cells by Nanosensors

Rapid and effective differentiation between normal and cancer cells is an important challenge for the diagnosis and treatment of tumors. A nanoparticle array-based

system has been described for identification of normal and cancer cells based on a "chemical nose/tongue" approach that exploits subtle changes in the physicochemical nature of different cell surfaces (Bajaj et al. 2009). Differential interactions with functionalized nanoparticles are transduced through displacement of a multivalent polymer fluorophore that is quenched when bound to the particle and fluorescent after release. This sensing method can rapidly (minutes/seconds) and effectively distinguish (1) different cell types; (2) normal, cancerous, and metastatic human breast cells; and (3) isogenic normal, cancerous, and metastatic murine epithelial cell lines.

9.2.4 Gold Nanoparticles for Cancer Diagnosis

Gold nanoparticles conjugated to anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibodies (MAbs) specifically and homogeneously bind to the surface of the cancer cells with 600 % greater affinity than to the noncancerous cells. This specific and homogeneous binding is found to give a relatively sharper surface plasmon resonance (SPR) absorption band with a red shifted maximum compared to that observed when added to the noncancerous cells (El-Sayed et al. 2005). The particles that worked the best were 35 nm in size. These results suggest that SPR scattering imaging or SPR absorption spectroscopy generated from antibody-conjugated gold nanoparticles can be useful in molecular biosensor techniques for the diagnosis and investigation of living oral epithelial cancer cells in vivo and in vitro. Advantages of this technique are:

- It is not toxic to human cells. A similar technique with QDs uses semiconductor crystals to mark cancer cells, but the semiconductor material is potentially toxic to the cells and humans.
- It does not require expensive high-powered microscopes or lasers to view the results. All it takes is a simple, inexpensive microscope and white light.
- The results are instantaneous. If a cancerous tissue is sprayed with gold nanoparticles containing the antibody, the results can be seen immediately. The scattering is so strong that a single particle can be detected.

An animal study has successfully demonstrated the safety of diagnostic use of Raman–silica–gold nanoparticles (R–Si–Au NPs), which overcome the inherently weak nature of Raman effect by producing larger Raman signals through surface-enhanced Raman scattering (Thakor et al. 2011). R–Si–Au NPs were bound to poly(ethylene glycol) (PEG) molecules to improve biological tolerance. Molecules that home in on cancer cells can be attached to PEG–R–Si–Au NPs, and the overall diameter is 100 nm. Photoimaging with these nanoparticles holds the promise of very early disease detection in colorectal cancer (CRC), even before any gross anatomical changes show up, without physically removing any tissue from the patient. Both rectal and intravenous administration of the particles did not show any systemic toxicity in experimental animals. Furthermore, the nanoparticles were quickly excreted. The intravenously administered nanoparticles were rapidly sequestered by scavenger cells resident in organs such as the liver and spleen. This opens the door

to human tests of intravenous injections of these nanoparticles to search for tumors throughout the body. Molecules targeting breast, lung, or prostate cancer can be attached to these nanoparticles. The investigators are now filing for Food and Drug Administration (FDA) approval to proceed to clinical studies of the nanoparticles for the diagnosis of CRC.

Chemiluminescence resonance energy transfer (CRET) with gold nanoparticles (AuNPs) has been used as an efficient long-range energy acceptor in sandwich immunoassays. A CRET-based sandwich immunoassay has been developed for alpha-fetoprotein (AFP) cancer biomarker (Huang and Ren 2011). In immunoassay, two antibodies (anti-AFP-1 and anti-AFP-2) are conjugated to AuNPs and horseradish peroxidase, respectively. The sandwich-type immunoreactions between the AFP (antigen) and the two different antibodies bridge the donors (luminol) and acceptors (AuNPs), which leads to the occurrence of CRET from luminol to AuNPs upon chemiluminescent reaction. We observed that the quenching of chemiluminescence signal depended linearly on the AFP concentration within a range of concentration from 5 to 70 ng mL^{-1}, and the detection limit of AFP was 2.5 ng mL^{-1}. This method was successfully applied for determination of AFP levels in sera from cancer patients, and the results were in good agreement with ELISA assays. This approach is expected to be extended to other assay designs, that is, using other antibodies, analytes, and chemiluminescent substance.

Gold nanoparticles can be heated rapidly whenever exposed to infrared light of the right wavelength. Heating of gold nanoparticles results in variations in pressure surrounding them, which in turn is expressed in the generation of ultrasound—a phenomenon called plasmon resonance. The shape of the particles determines the wavelength at which this happens. In this way, light from a laser results in sound. By attaching MAbs to gold nanoparticles or nanorods, which can recognize a specific cancer cell, the heating phenomenon can be used in cancer detection. This acoustic signal gives valuable information about the presence of cancer cells. Scientists at the University of Twente (UT) in the Netherlands expect better results with this approach than is currently possible with imaging techniques. The temperature rise can be up to 100 °C. Photothermal therapy would use the heated gold to destroy the tumor. Another option would be to include gold particles in capsules filled with cancer medication: the capsule attaches to the cancer cell and is heated, and the medicine is released locally. Both diagnostic and therapeutic applications will be investigated by the UT scientists together with colleagues from the Erasmus Medical Center in Rotterdam and two companies: Esaote Europe and Luminostix.

9.2.5 Gold Nanorods for Detection of Metastatic Tumor Cells

Scientists at Purdue University have developed a technique for producing biocompatible gold nanorods of various sizes to which antibodies can be attached. Gold nanorods interact with light to produce plasmons, a wavelike motion of electrons on the surface of the nanorods. Depending on the ratio of a nanorod's length to its

diameter, these plasmons trigger light emission at a specific frequency that is easily detected using SPR spectroscopy. An antibody that recognizes one specific cancer cell surface biomarker is attached to each nanorod of a given length and diameter. A gold nanorod–antibody construct that recognizes a biomarker found on all cell surfaces serves as an internal reference control that enables calculation of relative amounts of the various tumor biomarkers on a given cancer cell. Using a panel of three different antibody-labeled gold nanorods, the investigators were able to characterize breast tumors according to their cellular composition and correlate their findings to the metastatic potential of each given cell type. These results were validated using flow cytometry, the standard technique used to classify cells according to surface biomarkers. Gold nanorods enabled monitoring of as many as 15 different nanorod–antibody constructs simultaneously.

9.2.6 Implanted Biosensor for Cancer

An implant for biosensing of cancer, developed at the Massachusetts Institute of Technology (Cambridge, MA), contains nanoparticles that can be designed to test for different substances, including metabolites such as glucose and oxygen that are associated with tumor growth. It can also be used to test the effects of anticancer drugs in individual patients; the implant could reveal how much of a drug has reached the tumor. The nanoparticles are encased in a silicone delivery device, enabling their retention in patients' bodies for an extended period of time. The device can be implanted directly into a tumor, allowing a more direct look at what is happening in the tumor over a period of time. The technique makes use of detection nanoparticles composed of iron oxide and coated with dextran. Antibodies specific to the target molecules are attached to the surface of the nanoparticles. When the target molecules are present, they bind to the particles and cause them to clump together. That clumping can be detected by MRI. The nanoparticles are trapped inside the silicone device, which is sealed off by a porous membrane. The membrane allows molecules smaller than 30 nm to get in, but the detection particles are too large to get out. In addition to monitoring the presence of chemotherapy drugs, the device could also be used to check whether a tumor is growing or shrinking or whether it has spread to other locations, by sensing the amount and location of tumor biomarkers. Preclinical testing is being done for this device for human chorionic gonadotropin that can be considered a biomarker for cancer because it is produced by tumors but not normally found in healthy individuals except pregnant women.

9.2.7 Nanotubes for Detection of Cancer Proteins

Single-wall carbon nanotubes (SWCNTs) are being developed for monitoring cancer-specific proteins. These are hundreds of times smaller than nanocantilevers,

highly sensitive to single-protein binding events, and can be massively multiplexed with millions of tubes per chip for proteomic profiling. The tubes have extraordinary strength, unique electronic properties, and the ability to tag cancer-specific proteins to their surface. These tubes can be fabricated by decomposition of carbon-based gas in a furnace, using iron nanoparticles as catalyst material. With diameter of 1 nm and length of 1 μm, these tubes are smaller than a single strand of DNA. In other words, such a tube is an atomic arrangement of one layer of carbon atoms, which are on the surface. Protein binding events occurring on the surface of these tubes produce a measurable change in the mechanical and electrical properties.

By coating the surfaces of SWCNTs MAbs, it is possible to detect CTCs in the blood. SWCNTs covered with MAbs, particularly those for insulin-like growth factor-1 receptor (IGF-1), which is commonly found at high levels on cancer cells, home in on target protein "antigens" on the surface of CTCs. This method can be used for detection of recurring CTCs or residual micrometastases from the originally treated tumor. The technique could be cost-effective and could diagnose whether cells are cancerous or not in seconds versus hours or days required for conventional histology examination. It will enable large-scale production methods to make thousands of biosensors and have microarrays of these to detect the fingerprints of specific kinds of CTCs. Eventually it may be possible to design an assay that can detect CTCs on a handheld device no bigger than a cell phone. Limitation of the technique is that it may not detect more than one antigen at a time on a single cell.

9.2.7.1 Nanobiochip Sensor Technique for Analysis of Oral Cancer Biomarkers

A pilot study has described a nanobiochip sensor technique for analysis of oral cancer biomarkers in exfoliative cytology specimens, targeting both biochemical and morphologic changes associated with early oral tumorigenesis (Weigum et al. 2010). Oral lesions from dental patients, along with normal epithelium from healthy volunteers, were sampled using a noninvasive brush biopsy technique. Specimens were enriched, immunolabeled, and imaged in the nanobiochip sensor according to previously established assays for the epidermal growth factor receptor (EGFR) biomarker and cytomorphometry. Four key parameters were significantly elevated in both dysplastic and malignant lesions relative to healthy oral epithelium, including the nuclear area and diameter, the nuclear-to-cytoplasmic ratio, and EGFR biomarker expression. Further examination using logistic regression and receiver operating characteristic curve analyses identified morphologic features as the best predictors of disease individually, whereas a combination of all features further enhanced discrimination of oral cancer and precancerous conditions with high sensitivity and specificity. Further clinical trials are necessary to validate the regression model and evaluate other potential biomarkers. Nanobiochip sensor technique is a promising tool for early detection of oral cancer, which could enhance patient survival.

9.2.7.2 Nanodots for Tracking Apoptosis in Cancer

Apoptosis is a hallmark effect triggered by anticancer drugs. Researchers at Seoul National University (Korea) have developed a biocompatible, fluorescent nanoparticle that could provide an early sign that apoptosis is occurring as a result of anticancer therapy (Yu et al. 2007). The team created their fluorescent surface-enhanced Raman spectroscopic (F-SERS) nanodots to boost the optical signal generated by typical, biocompatible fluorescent dyes. The nanodots consist of silver nanoparticles embedded in a silica sphere. Attached to the silica core are fluorescent dye molecules and molecules known as Raman labels that enhance the electronic interactions between the silver nanoparticles and the dye molecules. The researchers also linked annexin V, a molecule that binds specifically to a chemical that appears on cells undergoing apoptosis, to the silica–silver nanoparticle construct. Toxicity tests showed that the silica–silver nanodots were not toxic to various human cells growing in culture. The investigators then added the nanodots to cells triggered to undergo apoptosis and were able to image those cells as they went through programmed cell death. Based on these results, the researchers prepared other nanodots containing antibodies that bind to other molecules involved in apoptosis. They then added these antibody-linked nanodots and the annexin V-linked nanodots to cultured human lung cancer cells. The investigators were able to track the appearance of all three molecules simultaneously, which has been difficult to do using conventional cell staining techniques.

9.2.7.3 Nanolaser Spectroscopy for Detection of Cancer in Single Cells

Nanolaser scanning confocal spectroscopy can be used to identify a previously unknown property of certain cancer cells that distinguishes them with single-cell resolution from closely related normal cells. This property is the correlation of light scattering and spatial organization of mitochondria; normally it is well scattered, but in cancer cells, the mitochondria are disorganized and scatter light poorly. These optical methods are promising powerful tools for detecting cancer at an early stage.

9.2.7.4 Nanoparticles Designed for Dual-Mode Imaging of Cancer

The best characteristics of QDs and magnetic iron oxide nanoparticles have been combined to create a single-nanoparticle probe that can yield clinically useful images of both tumors and the molecules involved in cancer (Choi et al. 2006). The authors started by synthesizing 30-nm-diameter silica nanoparticles impregnated with rhodamine, a bright fluorescent dye, and 9-nm-diameter water-soluble iron oxide nanoparticles. They then mixed these two nanoparticles with a chemical linker, yielding the dual-mode nanoparticle. On average, ten magnetic iron oxide particles link to a single dye-containing silica nanoparticle, and the resulting construct is ~45 nm in diameter. The combination nanoparticle performed better in both

MRI and fluorescent imaging tests than did the individual components. In MRI experiments, the combination nanoparticle generated an MRI signal that was over threefold more intense than did the same number of iron oxide nanoparticles. Similarly, the fluorescent signal from the dual-mode nanoparticle was almost twice as bright as that produced by dye molecules linked directly to iron oxide nanoparticles. Next, the researchers labeled the dual-mode nanoparticles with an antibody that binds to molecules known as polysialic acids, which are found on the surface of certain nerve cell and lung tumors. These targeted nanoparticles were quickly taken up by cultured tumor cells and were readily visible using fluorescence microscopy.

9.2.7.5 Nanotechnology-Based Single-Molecule Assays for Cancer

Information about the biological processes in living cells is required for the detection and diagnosis of cancer for the following reasons:

- To recognize the important changes that occur when cells undergo malignant transformation.
- There are situations when primary cells from a surgical procedure cannot be propagated due to the type of cell or the low number of cells available.
- Detection of cancer at an early stage is a critical step for improving cancer treatment.

Early detection will require sensitive methods for isolating and interrogating individual cells with high spatial and temporal resolution without disrupting their cellular biochemistry. Probes designed to penetrate a cell and report on the conditions within that cell must be sufficiently small, exceedingly bright, and stable for a long time in the intracellular environment without disrupting the cell's normal biochemical functioning. A series of silver nanoparticles have been prepared that meet many of the criteria listed above. Although smaller than 100 nm in diameter, these particles are bright enough to be seen by eye using optical microscopy. Unlike fluorophores, fluorescent proteins, or quantum dots, silver nanoparticles do not photodecompose during extended illumination. Therefore, they can be used as a probe to continuously monitor dynamic events in living cells during studies that last for weeks or even months. Because the color of the scattered light from nanoparticles depends upon their size, they have been used to measure the change in single-membrane pores in real time using dark-field optical microscopy. Intracellular and extracellular nanoparticles can also be differentiated by the intensity of light scattering. Next challenge is to develop methods for modifying the surface of the nanoparticles to make them more biocompatible, so that biological processes can be observed without disturbing or destroying the cell's intrinsic biochemical machinery. Ultimately, these probes may be combined to produce highly sensitive assays with high spatial and temporal resolution. This advance will enable researchers to study the interactions of multiple genes in the same cell simultaneously by using different colored reporter molecules. In addition to transcription and translation, similar live-cell single-molecule assays will offer the prospect of studying more

complex cellular processes, such as cell signaling. Continuous advances and evolution along these research fronts are necessary to unravel biochemical processes in vivo and to develop tools that can be used to detect and diagnose cancer using only a single cell from the patient.

9.2.7.6 QDs for Detection of Tumors

QD bioconjugates that are highly luminescent and stable can be used for studying gene and enable visualization of cancer cells in living animals. QDs can be combined with fluorescence microscopy to follow cells at high resolution in living animals. These offer considerable advantages over organic fluorophores for this purpose. QDs and emission spectrum scanning multiphoton microscopy have been used to develop a means to study extravasation of tumor cells in vivo.

9.2.7.7 QD-Based Test for DNA Methylation

DNA methylation contributes to carcinogenesis by silencing key tumor suppressor genes. An ultrasensitive and reliable nanotechnology assay, MS-qFRET (fluorescence resonance energy transfer), can detect and quantify DNA methylation (Bailey et al. 2009). Bisulfite-modified DNA is subjected to PCR amplification with primers that would differentiate between methylated and unmethylated DNA. QDs are then used to capture PCR amplicons and determine the methylation status via FRET. The specific target of the test is DNA methylation which occurs when methyl attaches to cytosine, a DNA building block. When this happens at specific gene locations, it can stop the release of tumor-suppressing proteins; cancer cells then more easily form and multiply. The method involves singling out the DNA strands with methyl attachments through bisulfite conversion, whereby all non-methyl segments are converted into another nucleotide. Copies of the remaining DNA strands are made, two molecules (a biotin protein and a fluorescent dye) are attached at either end, and the strands are mixed with QDs that are coated with a biotin-attractive chemical. Up to 60 DNA strands are attracted to a single QD. An UV light or blue laser activates the QDs, which pass the energy to the fluorescent molecules on the DNA strands which then light up and are identifiable via a spectrophotometer, which both identifies and can count the DNA methylation.

Key features of MS-qFRET include its low intrinsic background noise, high resolution, and high sensitivity. This approach detects as little as 15 pg of methylated DNA in the presence of a 10,000-fold excess of unmethylated alleles, enables reduced use of PCR (as low as eight cycles), and allows for multiplexed analyses. The high sensitivity of MS-qFRET enables one-step detection of methylation at PYCARD, CDKN2B, and CDKN2A genes in patient sputum samples that contain low concentrations of methylated DNA, which normally would require a nested PCR approach.

The direct application of MS-qFRET on clinical samples offers great promise for its translational use in early cancer diagnosis and prognostic assessment of tumor

behavior, as well as monitoring response to therapeutic agents. Gene DNA methylation indicates a higher risk of developing cancer and is also seen as a warning sign of genetic mutations that lead to development of cancer. Moreover, since different cancer types possess different genetic markers, e.g., lung cancer biomarkers differ from those of leukemia, the test should identify which cancer a patient is at risk of developing. This test could be used for frequent screening for cancer and replacing traditionally invasive methods with a simple blood test. It could also help determine whether a cancer treatment is effective and thus enable personalized chemotherapy.

9.2.8 Nanobiotechnology for Early Detection of Cancer to Improve Treatment

Cancer cells themselves may be difficult to detect at an early stage, but they leave a fingerprint, i.e., a pattern of change in biomarker proteins that circulate in the blood. There may be 20–25 biomarkers, which may require as many as 500 measurements, all of which should be made from a drop of blood obtained by pinprick. Thus nanoscale diagnostics will play an important role in this effort. Nanowire sensors are in development at California Institute of Technology (Pasadena, CA) for very early diagnosis of cancer, when there are just a few thousand cells. Nanowires can electronically detect a few protein molecules along with other biochemical markers that are early signs of cancer. Nanowires in a set are coated with several compounds, each of which binds to a particular biomarker and changes the conductivity of the nanowire that can be measured. Thousands of such nanowires are combined on a single chip that enables identification of the type of cancer. Currently such a chip can detect between 20 and 30 biomarkers and is being used for the early diagnosis of brain cancer.

Cancer is easier to treat and less likely to develop drug resistance when treatment is started very early. Cancer cells in very early stages are less likely to have mutations that make them resistant to treatment.

An automated gold nanoparticle bio-barcode assay probe has been described for the detection of prostate-specific antigen (PSA) at 330 fg/mL, along with the results of a clinical pilot study designed to assess the ability of the assay to detect PSA in the serum of 18 men who have undergone radical prostatectomy for prostate cancer (Thaxton et al. 2009). Available PSA immunoassays are often not capable of detecting PSA in the serum of men after radical prostatectomy. This new bio-barcode PSA assay is approximately 300 times more sensitive than commercial immunoassays, and all patients in this study had a measurable serum PSA level after radical prostatectomy. Because the patient outcome depends on the level of PSA, this ultrasensitive assay enables (1) informing patients, who have undetectable PSA levels with conventional assays but detectable and nonrising levels with the barcode assay, that their cancer will not recur; (2) earlier detection of recurrence because of the ability to measure increasing levels of PSA before conventional tools can make such assignments; and (3) use of PSA levels, which would otherwise not be detectable with conventional assays, to follow the response of patients to treatment.

9.3 Nanobiotechnology-Based Drug Delivery in Cancer

Drug delivery in cancer is important for optimizing the effect of drugs and reducing toxic side effects. Several nanobiotechnologies, mostly based on nanoparticles, have been used to facilitate drug delivery in cancer. A classification of the nanotechnologies for drug delivery in cancer is shown in Table 9.1.

Approximately 150 drugs in development for cancer are based on nanotechnology. Those approved are listed in Table 9.2 and several more are in clinical trials.

Table 9.1 Classification of nanobiotechnology approaches to drug delivery in cancer

Nanoparticles
Nanoparticle formulations of anticancer drugs, e.g., paclitaxel
Exosomes for cancer drug delivery
Nanoencapsulation and enclosure of anticancer drugs
Enclosing drugs in lipid nanocapsules
Encapsulating drugs in hydrogel nanoparticles
Micelles for drug delivery in cancer
Targeted delivery of anticancer therapy
Targeted drug delivery with nanoparticles
PEGylated nanoliposomal formulation
Folate-linked nanoparticles
Carbon magnetic nanoparticles for targeted drug delivery in cancer
Targeted drug delivery with nanoparticle–aptamer bioconjugates
Nanodroplets for site-specific cancer treatment
Lipid-based nanocarriers
Targeted antiangiogenic therapy using nanoparticles
Nanoparticles for delivery of drugs to brain tumors
Combination of nanoparticles with radiotherapy
Combination with boron neutron capture therapy
Nanoengineered silicon for brachytherapy
Combination with physical modalities of cancer therapy
Combination with laser ablation of tumors
Combination with photodynamic therapy
Combination with thermal ablation
Combination with ultrasound
Nanoparticle-mediated gene therapy
p53 gene therapy of cancer
Immunolipoplex for delivery of p53 gene
Intravenous delivery of FUS1 gene
Strategies combining diagnostics and therapeutics
Nanoshells as adjuncts to thermal tumor ablation
Perfluorocarbon nanoparticles
Nanocomposite devices

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Table 9.2 Approved anticancer drugs using nanocarriers

Trade name/compound	Manufacturer	Nanocarrier
Abraxane/paclitaxel	Abraxis Bioscience	Albumin-bound paclitaxel
Bexxar/anti-CD20 conjugated to iodine-131	Corixa/GlaxoSmithKline	Radioimmunoconjugate
DaunoXome/daunorubicin	Diatos, available in France	Liposome
Doxil/Caelyx/doxorubicin	Ortho Biotech	Liposome
Myoset/doxorubicin	Cephalon, available in Europe	Non-PEGylated liposome
Oncaspar/PEG-L-asparaginase	Enzon	Polymer–protein conjugate
Ontak/IL-2 fused to diphtheria toxin	Eisai Inc.	Immunotoxic fusion protein
SMANCS/zinostatin	Yamanouchi Pharmaceutical Co	Polymer–protein conjugate
Zevalin/anti-CD20 conjugated to yttrium-90	Cell Therapeutics Inc.	Radioimmunoconjugate
Zoladex/goserelin acetate	AstraZeneca	Polymer rods

© Jain PharmaBiotech
SMANCS styrene maleic anhydride neocarzinostatin

9.3.1 Nanoparticle Formulations for Drug Delivery in Cancer

9.3.1.1 Anticancer Drug Particles Incorporated in Liposomes

Several injectable and biodegradable systems have been synthesized based on incorporation of anti-estrogens (AEs) in nanoparticles and liposomes. Both nanospheres and nanocapsules (polymers with an oily core in which AEs were solubilized) incorporated high amounts of 4-hydroxytamoxifen (4-HT) or RU 58668. Liposomes containing various ratios of lipids enhanced the apoptotic activity of RU 58668 in several multiple myeloma cell lines tested by flow cytometry. These cell lines expressed both estrogen receptor alpha and beta subtypes. RU-loaded liposomes, administered intravenously in an animal model, induce the arrest of tumor growth. Thus, the drug delivery of anti-estrogens enhances their ability to arrest the growth of tumors which express estrogen receptors and are of particular interest for estrogen-dependent breast cancer treatment. In addition, it represents a new potent therapeutic approach for multiple myeloma.

SuperFluids™ technology (Aphios Corporation) involves biodegradable polymer nanospheres utilizing supercritical, critical, or near-critical fluids with or without polar cosolvents. These nanospheres are utilized to encapsulate proteins with controlled release characteristics without usage of toxic organic solvent. The patented technology can be utilized to form stable biocompatible aqueous formulations of poorly soluble anticancer drugs such as paclitaxel and camptothecin (CPT). An improved process utilizing SuperFluids™ results in the formation of small, uniform liposomes (nanosomes) to improve the delivery and therapeutic efficacy of poorly water-soluble drugs while reducing their toxicities. The process has been used for the

nanoencapsulation of paclitaxel in a formulation called Taxosomes™, which has been tested in nude mice with breast cancer xenografts. Taxosomes™ will lead to (1) enhanced therapeutic efficacy; (2) elimination of premedication to counteract castor oil; (3) reduction of drug toxicity side effects; (4) prolonged circulation time and therapeutic effect; and (5) improved quality of life.

The process has also been used for the nanoencapsulation of CPT, a potent and exciting anticancer agent, in a stable aqueous liposomal formulation called Camposomes™. Water-soluble derivatives of CPT, a unique topoisomerase 1 inhibitor, have recently been approved by the FDA for use in CRC. Camposomes™ have been shown to be very effective against lymphomas in nude mice.

Nanocapsules, which are small aggregates of cisplatin covered by a single lipid bilayer, have an unprecedented drug-to-lipid ratio and an in vitro cytotoxicity up to 1,000-fold higher than the free drug. Analysis of the mechanism of nanocapsule formation suggests that the method may be generalized to other drugs showing low water solubility and lipophilicity.

In Protein Stabilized Liposome (PSL™) nanotechnology of Azaya Therapeutics, the liposome product is prepared in a single step that encapsulates the active drug docetaxel (ATI-1123) in the lipid layer of the liposome while forming active nanoparticles in situ (100–130 nm). This process is geared toward the formulation of hydrophobic molecules that would otherwise have limited success as developmental drugs using traditional formulation methodologies. Azaya intends to use its PSL nanotechnology to improve the performance and reduce the nonspecific cytotoxicity of leading marketed chemotherapeutics such as Taxotere (docetaxel) and CAMPTOSAR®, as well as several experimental drugs that have been withdrawn from development due to their nonspecific cytotoxicity and formulation difficulties.

9.3.1.2 Cerasomes

Ceramide is a lipid molecule in plasma membrane of the cell and controls cell functions such as cell aging. Ceramide selectively kills cancer cells but is not toxic to normal cells. However, as a lipid, ceramide cannot be delivered effectively as a drug. To solve this limitation, cerasome is created to turn the insoluble lipid into a soluble form. Cerasomes are molecular-sized bubbles (size range 60–200 nm) filled with C6-ceramide for use as anticancer agents. Paclitaxel-loaded cerasomes exhibit sophisticated controlled release behavior for drug delivery in cancer (Cao et al. 2010). Cerasomes have already been shown to effectively treat cellular and animal models of breast cancer and melanoma. Systemic administration of nanoliposomal C6-ceramide to mice engrafted with SK-HEP-1 tumors reduced tumor vascularization and proliferation, induced tumor cell apoptosis, decreased phosphorylation of AKT, and ultimately blocked tumor growth (Tagaram et al. 2011). These studies show that nanoliposomal ceramide is an efficacious antineoplastic agent for the treatment of in vitro and in vivo models of human hepatocellular carcinoma.

9.3.1.3 Encapsulating Drugs in Polymeric Nanoparticles

Curcumin, an element found in the cooking spice turmeric, has long been known to have potent anticancer properties as demonstrated in several human cancer cell lines and animal carcinogenesis models. Nevertheless, widespread clinical application of this relatively efficacious agent in cancer and other diseases has been limited due to poor aqueous solubility, and consequently, minimal systemic bioavailability. This problem has been overcome by encapsulating free curcumin with a polymeric nanoparticle (PNP), creating nanocurcumin (Bisht et al. 2007). Further, nanocurcumin's mechanisms of action on pancreatic cancer cells mirror that of free curcumin, including induction of cellular apoptosis, blockade of nuclear factor kappa B (NF-κB) activation, and downregulation of steady-state levels of multiple proinflammatory cytokines (IL-6, IL-8, and TNF-α). No evidence of toxicity was found in tests with empty versions of the PNP. Their findings show no evidence of weight loss, organ changes, or behavioral changes in live mice after administering a relatively large dosage of the empty nanoparticles. Nanocurcumin provides an opportunity to expand the clinical repertoire of this efficacious agent by enabling ready aqueous dispersion. Future studies utilizing nanocurcumin are warranted in preclinical in vivo models of cancer and other diseases that might benefit from the effects of curcumin.

9.3.1.4 Encapsulating Drugs in Hydrogel Nanoparticles

A versatile chemical technique has been developed for creating ultrafine nanosized hydrogels, essentially a network of polymer chains that absorb as much as 99 % of their weight in water (Gao et al. 2007). Polyacrylamide was used to create nanoparticles of 2-nm diameter that have no charge on their surfaces. This lack of charge prevents blood proteins from sticking to the surface of the nanoparticles. Combined with the fact that these nanoparticles are too small to be recognized by the immune system, the result is a nanoscale drug delivery vehicle with the ability to remain in circulation long enough to reach and permeate tumors before being excreted through the kidneys. These nanoscale hydrogels were first tested as a drug delivery vehicle for a water-insoluble photosensitizer called meta-tetra(hydroxyphenyl)chlorin (mTHPC), which is approved in the European Union for use in treating head and neck cancer. mTHPC produces cell-killing reactive oxygen when irradiated with red light but not without serious side effects resulting from the method now used to deliver this drug to tumors. When added to the chemical mixture used to create the nanoparticles, mTHPC becomes trapped within the polymer framework. Characterization experiments showed that this photosensitizer does not escape from the nanoparticles but is still capable of producing the same amount of reactive oxygen as if it were free in solution. When added to human brain cancer cells growing in culture and irradiated with red light, this formulation kills the cells rapidly. Empty nanoparticles had no effect on the cells. Neither did drug-loaded nanoparticles added to the cells that were kept in the dark.

9.3.1.5 Exosomes

Exosomes are small (50–100 nm), spherical vesicles produced and released by most cells to facilitate intercellular communication. These vesicles are of endosomal origin and are secreted in the extracellular milieu following fusion of late endosomal multivesicular bodies with the plasma membrane. Exosomes have a defined protein composition, which confers specific biological activities contingent on the nature of the producing cell. Although exosomes express tumor antigens, leading to their proposed utility as tumor vaccines, they also can suppress T-cell signaling molecules and induce.

Exosomes produced by dendritic cells are called dexosomes and contain essential components to activate both adaptive and innate immune responses. Anosys is developing dexosome vaccines that use patient-specific dexosomes loaded with tumor antigen-derived peptides to treat cancer. Exosome research continues to reveal unique properties which broaden their fields of application. Anosys' Exosome Display Technology provides the ability to manipulate exosome composition and tailor exosomes with new desirable properties opening up opportunities in the field of recombinant vaccine and MAb preparation. This is achieved by generating genes coding for chimeric proteins linking an exosome addressing sequence to antigens or biologically active proteins. The resulting proteins are targeted to exosomal compartment and released in the extracellular milieu bound to exosomes.

Exosomes are emerging as novel approaches for cancer vaccine development. Safety of exosomes has been established in clinical trials that can be administered, but their potency for eliciting appropriate immune responses to kill cancer cells leaves much to be desired (Tan et al. 2010). Most of the investigational evidence is about solid tumors, and it has not been demonstrated that nonsolid tumors (e.g., hematological malignancies) can be treated using exosome technology. Moreover, exosomal immunotherapy relies on the immune system, and cancer patients, who are immunocompromised and/or immunosuppressed due to chemotherapy and radiotherapy, might not be able to overcome cancer with their immune system alone.

9.3.1.6 Folate-Linked Nanoparticles

PEG-coated biodegradable nanoparticles can be coupled to folic acid to target the folate-binding protein; this molecule is the soluble form of the folate receptor that is overexpressed on the surface of many tumor cells. The specific interaction between the conjugate folate nanoparticles and the folate-binding protein has been evaluated by surface plasmon resonance and confirmed a specific binding of the folate nanoparticles to the folate-binding protein. Thus, folate-linked nanoparticles represent a potential new drug carrier for tumor cell-selective targeting.

9.3.1.7 Iron Oxide Nanoparticles

A novel water-dispersible oleic acid (OA)–Pluronic-coated iron oxide magnetic nanoparticle formulation – can be loaded easily with high doses of water-insoluble anticancer agents (Jain et al. 2005). Drug partitions into the OA shell surrounding iron oxide nanoparticles and the Pluronic that anchors at the OA–water interface confer aqueous dispersity to the formulation. Neither the formulation components nor the drug loading affects the magnetic properties of the core iron oxide nanoparticles. Sustained release of the incorporated drug is observed over 2 weeks under in vitro conditions. The nanoparticles have further demonstrated sustained intracellular drug retention relative to drug in solution and a dose-dependent antiproliferative effect in breast and prostate cancer cell lines. This nanoparticle formulation can be used as a universal drug carrier system for systemic administration of water-insoluble drugs while simultaneously enabling magnetic targeting and/or imaging.

9.3.1.8 Lipid-Based Nanocarriers

LiPlasome Pharma's proprietary prodrug and drug delivery technology is based on smart lipid-based nanocarriers (LiPlasomes) that can be applied for targeted transport of anticancer drugs (Anderson et al. 2005). The targeted drug delivery principle consists of long-circulating nanoparticles such as liposomes or micelles that accumulate in porous cancer tissue with a high PLA2 activity. The carrier nanoparticles are composed of special prodrug lipids whose degradation products, after exposure to PLA2, are converted to active drugs such as anticancer lysolipids and/or fatty acid drug derivatives. The PLA2 hydrolysis products will furthermore act as locally generated permeability enhancers that promote the absorption of the released drugs across the cancer cell membranes into putative intracellular target sites. This innovative prodrug and drug delivery concept allows for intravenous transport of high concentrations of anticancer drugs directly to the tumor target. It enables, without any prior knowledge of the position and size of the tumor, to release the anticancer drugs specifically at the tumor target site. The delivery system is formulated with PEG to prolong the serum half-life of the drugs and prodrugs and avoid the nanocarriers being removed by the reticuloendothelial system.

9.3.1.9 Micelles for Drug Delivery in Cancer

Block copolymer micelles are spherical supramolecular assemblies of amphiphilic copolymers that have a core-shell-type architecture. The core is a loading space that can accommodate hydrophobic drugs, and the shell is a hydrophilic brushlike corona that makes the micelle water soluble, thereby allowing delivery of the poorly soluble contents (Fig. 9.1).

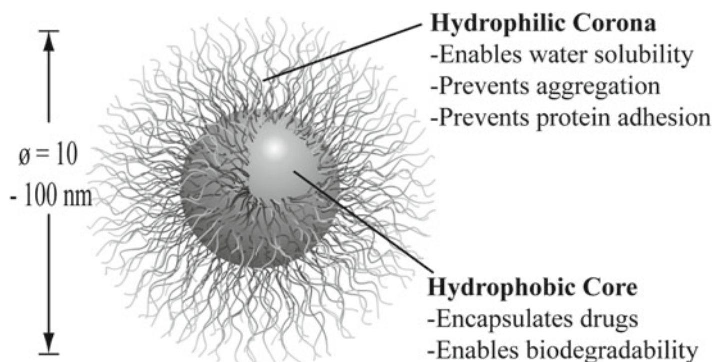

Fig. 9.1 Use of micelles for drug delivery

However, a key issue with the contained cytotoxic drugs is an understanding of how the micelle and the micelle-incorporated agent are distributed. By using fluorescently labeled polymer and organelle-specific dyes in combination with confocal microscopy, it has been shown that the micelles localize in several cytoplasmic organelles, including the mitochondria, but not the nucleus. Furthermore, the micelles increase the amount of a drug delivered to the cells and have the potential to deliver drugs to particular subcellular targets. Antibodies can be attached to the polymers that make up the micelles. Paclitaxel micelle (NanoCarrier Ltd), a tumor-targeted drug delivery system, has been investigated in clinical trials by Nippon Kayaku Co in Japan and is entering phase III in 2012. These micelles have a size of ~28 nm and exhibit a sustained drug release accompanied with the decay of the carrier itself in physiological saline. They show remarkably prolonged blood circulation and effectively accumulate in solid tumors indicating a potential for the targeted therapy of solid tumors.

DACH-platin-PEG-polyglutamic acid (DACH Platin Medicelle™) from NanoCarrier, based on Medicelle™ technology, has demonstrated enhanced permeability and retention (EPR) of the compound in the tumor, leading to improved efficacy and toxicity profiles in animal experiments. The mechanism of action of Medicelle™ delivery system is based on the formation of micelles, including hydrophilic–hydrophobic block copolymers, with a hydrophobic inner core and hydrophilic outer shell. This allows the chemical entrapment of various drugs into the micelles. The drugs are then released slowly into the organism. This product is being developed for clinical application.

CPT is a topoisomerase I inhibitor that is effective against cancer, but clinical application of CPT is limited by insolubility, instability, and toxicity problems. Biocompatible, targeted sterically stabilized micelles (SSMs) have been used as nanocarriers for CPT (CPT–SSM). CPT solubilization in SSM is reproducible and is attributed to avoidance of drug aggregate formation. Furthermore, SSMs composed

of polyethylene glycol (PEGylated) phospholipids are attractive nanocarriers for CPT delivery because they are sufficiently small (~14 nm) to extravasate through the leaky microvasculature of tumor and inflamed tissues for passive targeting of solid cancers in vivo, resulting in high drug concentration in tumors and reduced drug toxicity to the normal tissues (Koo et al. 2006).

Stealth micelle formulations have stabilizing PEG coronas to minimize opsonization of the micelles and maximize blood circulation times. Clinical data have been reported on three stealth micelle systems: SP1049C, NK911, and Genexol-PM (Sutton et al. 2007). SP1049C is formulated as doxorubicin (DOX)-encapsulated Pluronic micelles, NK911 is DOX-encapsulated micelles from a copolymer of PEG and DOX-conjugated poly(aspartic acid), and Genexol-PM is a paclitaxel-encapsulated PEG–PLA micelle formulation. Polymer micelles are becoming a powerful nanotherapeutic platform that affords several advantages for targeted drug delivery in cancer, including increased drug solubility, prolonged circulation half-life, selective accumulation at tumor sites, and a decrease in toxicity.

9.3.1.10 Minicells for Targeted Delivery of Nanoscale Anticancer Therapeutics

Indiscriminate drug distribution and severe toxicity of systemic administration of chemotherapeutic agents can be overcome through encapsulation and cancer cell-specific targeting of chemotherapeutics in 400-nm minicells (EnGeneIC Delivery Vehicle). Targeted minicells enter the cancer cells via receptor-mediated endocytosis, while the bacteria-carrying nanoparticles enter the mammalian cells in a nonspecific manner, i.e., via phagocytosis. Scientists at EnGeneIC discovered that minicells can be packaged with therapeutically significant concentrations of chemotherapeutics of differing charge, hydrophobicity, and solubility (MacDiarmid et al. 2007). Targeting of minicells via bispecific antibodies to receptors on cancer cell membranes results in endocytosis, intracellular degradation, and drug release. Doses of drugs delivered via minicells are ~1,000 times less than the dose of the free drug required for equivalent or better tumor shrinkage. It produces significant tumor growth inhibition and regression in mouse xenografts and lymphoma in dogs despite administration of minute amounts of drug and antibody, a factor critical for limiting systemic toxicity that should allow the use of complex regimens of combination chemotherapy. Phase I clinical trials are in progress in Australia.

In a further study, minicells were shown to specifically and sequentially deliver to tumor xenografts siRNAs or shRNA-encoding plasmids to counteract drug resistance by knocking down a multidrug resistance (MDR) protein (MacDiarmid et al. 2009). Subsequent administration of targeted minicells containing cytotoxic drugs eliminates formerly drug-resistant tumors. The dual sequential treatment, involving minicells loaded with both types of payload, enables complete survival without toxicity in mice with tumor xenografts, while involving several 1,000-fold less drug, siRNA, and antibody than needed for conventional systemic administration of cancer therapies.

9.3.1.11 Nanocarriers Enhance Doxorubicin Uptake in Drug-Resistant Cancer

Resistance to anthracyclines and other chemotherapeutics due to P-glycoprotein (Pgp)-mediated export is a frequent problem in cancer treatment. Iron oxide–titanium dioxide (TiO_2) core-shell nanocomposites can serve as efficient carriers for doxorubicin to overcome this common mechanism of drug resistance in cancer cells (Arora et al. 2012). Doxorubicin nanocarriers (DNCs) increased effective drug uptake in drug-resistant ovarian cells. Doxorubicin binds to the TiO_2 surface by a labile bond that is severed upon acidification within cell endosomes. Upon its release, doxorubicin traverses the intracellular milieu and enters the cell nucleus by a route that evades Pgp-mediated drug export. Confocal and X-ray fluorescence microscopy and flow cytometry have been used to show the ability of DNCs to modulate transferrin uptake and distribution in cells. Increased transferrin uptake occurs through clathrin-mediated endocytosis, indicating that nanocomposites and DNCs may both interfere with removal of transferrin from cells. Together, these findings show that DNCs not only provide an alternative route of delivery of doxorubicin to Pgp-overexpressing cancer cells but also may boost the uptake of transferrin-tagged therapeutic agents.

9.3.1.12 Nanoconjugates for Subcutaneous Delivery of Anticancer Drugs

Most of the anticancer drugs are administered intravenously. Nanoformulations of anticancer drugs are being developed for subcutaneous delivery of existing and new chemotherapeutics. Subcutaneous nanocarrier delivery of hyaluronan-conjugated doxorubicin or cisplatin has demonstrated significantly improved efficacy with decreased toxicity compared with standard agent combination therapy at all doses tested, achieving complete pathologic tumor response in mice implanted with human tumors (Cohen et al. 2011). Advantages of subcutaneous anticancer drug delivery are:

- Avoids complicated and expensive intravenous infusions
- Improves safety and efficacy for existing chemotherapy drugs
- Highly localized drug delivery to primary tumor sites to prevent recurrence
- Enables development of multidrug combinations to overcome drug resistance
- Incorporation of imaging agents to monitor penetration of the drug into tumor

9.3.1.13 Nanomaterials for Delivery of Poorly Soluble Anticancer Drugs

Nanomaterials have been successfully manipulated to create a new drug delivery system that can solve the problem of poor water solubility of most promising currently available anticancer drugs and thereby increase their effectiveness. The poorly soluble anticancer drugs require the addition of solvents in order for them to be

easily absorbed into cancer cells. Unfortunately, these solvents not only dilute the potency of the drugs but create toxicity as well. A novel approach has been devised using silica-based nanoparticles to deliver the anticancer drug CPT and other water-insoluble drugs into human cancer cells (Lu et al. 2007). The method incorporates a hydrophobic anticancer drug CPT into the pores of fluorescent mesoporous silica nanoparticles (MSNs) and delivers the particles into a variety of human cancer cells to induce cell death. The results suggest that the MSNs might be used as a vehicle to overcome the insolubility problem of many anticancer drugs.

9.3.1.14 Nanoparticle Formulation for Enhancing Anticancer Efficacy of Cisplatin

Cisplatin is a first-line chemotherapy for most types of cancer. However, its use is dose-limited due to severe nephrotoxicity. Rational engineering of a novel nanoplatinate has been reported, which self-assembles into a nanoparticle at unique platinum-to-polymer ratio and releases cisplatin in a pH-dependent manner (Paraskar et al. 2010). The nanoparticles are rapidly internalized into the endolysosomal compartment of cancer cells and exhibit an IC50 comparable to that of free cisplatin and superior to carboplatin. The nanoparticles showed significantly improved anticancer efficacy in terms of tumor growth delay in breast and lung cancers. Furthermore, the nanoparticle treatment resulted in reduced systemic and nephrotoxicity, validated by decreased biodistribution of platinum to the kidney. Given the need for a better platinate, this coupling of nanotechnology and structure-activity relationship to rationally reengineer cisplatin is anticipated to have a major impact on the treatment of cancer.

9.3.1.15 Nanoparticle Formulations of Paclitaxel

Paclitaxel is active and widely used to treat multiple types of solid tumors. The commercially available paclitaxel formulation uses Cremophor/ethanol (C/E) as the solubilizers. Other formulations including nanoparticles have been introduced. A study evaluated the effects of nanoparticle formulation of paclitaxel on its tissue distribution in experimental animals (Yeh et al. 2005). The nanoparticle and C/E formulations showed significant differences in paclitaxel disposition; the nanoparticles yielded 40 % smaller area under the blood concentration–time curve and faster blood clearance of total paclitaxel concentrations (sum of free, protein-bound, and nanoparticle-entrapped drug). Tissue specificity of the two formulations was different. The nanoparticles showed longer retention and higher accumulation in organs and tissues, especially in the liver, small intestine, and kidney. The most striking difference was an eightfold greater drug accumulation and sustained retention in the kidney. These data indicate that nanoparticulate formulation of paclitaxel affects its clearance as well as distribution in tissues with preferential accumulation in the liver, spleen, small intestine, and kidney. Solid tumors have unique features, such as leaky tumor blood vessels and defective lymphatic drainage, that promote

the delivery and retention of macromolecules or particles, a phenomenon recognized as the EPR effect. Tissue specificity of the gelatin nanoparticles warrants further investigations before using nanoparticle formulations of anticancer drugs for tumors in various organs.

AI-850 (Acusphere Inc.) is a rapidly dissolving porous particle formulation of paclitaxel, created by using the company's Hydrophobic Drug Delivery Systems. The patented spray-drying technology embeds small drug particles inside hydrophobic water-soluble matrices so that the whole composition is a mixture of microparticles and nanoparticles. AI-850 was compared to Taxol following intravenous administration in a rat pharmacokinetic study, a rat tissue distribution study, and a human xenograft mammary tumor model in nude mice (Straub et al. 2005). The volume of distribution and clearance for paclitaxel following intravenous bolus administration of AI-850 were sevenfold and fourfold greater, respectively, than following intravenous bolus administration of Taxol. There were no significant differences between AI-850 and Taxol in tissue concentrations and area under the curve for the tissues examined. Nude mice implanted with mammary tumors showed improved tolerance of AI-850, enabling higher administrable dose of paclitaxel, which resulted in improved efficacy as compared to Taxol administered at its maximum tolerated dose.

Gold nanoparticles (2 nm) have been covalently functionalized with paclitaxel (Gibson et al. 2007). The synthetic strategy involves the attachment of a flexible hexaethylene glycol linker at the C-7 position of paclitaxel followed by coupling of the resulting linear analog to phenol-terminated gold nanocrystals. The reaction yields the product with a high molecular weight, while exhibiting an extremely low polydispersity index. The organic shell of hybrid nanoparticles contains 67 % by weight of paclitaxel, which corresponds to ~70 molecules of the drug per 1 nanoparticle. High-resolution transmission electron microscope (TEM) was employed for direct visualization of the inorganic core of hybrid nanoparticles, which were found to retain their average size, shape, and high crystallinity after multiple synthetic steps and purifications. The interparticle distance substantially increases after the attachment of paclitaxel as revealed by low-magnification TEM, suggesting the presence of a larger organic shell. Thus organic molecules with exceedingly complex structures can be covalently attached to gold nanocrystals in a controlled manner and fully characterized by traditional analytical techniques. In addition, this approach gives a rare opportunity to prepare hybrid particles with a well-defined amount of drug and offers a new alternative for the design of nanosized drug delivery systems. Follow-up studies will determine the potency of the paclitaxel-loaded nanoparticles. Since each ball is loaded with a uniform number of drug molecules, it will be relatively easy to compare the effectiveness of the nanoparticles with the effectiveness of generally administered paclitaxel. This technique could help to deliver more of the drug directly to the cancer cells and reduce the side effects of chemotherapy. The aim is to improve the effectiveness of the drug by increasing its ability to stay bound to microtubules within the cell.

Albumin nanoparticle technology enables the transportation of hydrophobic drugs such as paclitaxel without the need of potentially toxic solvents. Nab-paclitaxel can be administered without premedication, in a shorter infusion time and without the

need for a special infusion set. Moreover, this technology allows the selective delivery of larger amounts of anticancer drug to tumors, by exploiting endogenous albumin pathways. Nab-paclitaxel is approved for the treatment of metastatic breast cancer, after the failure of first-line standard therapy, when anthracyclines are not indicated. Efficacy and safety data, along with a more convenient administration, confirm the potential for nab-paclitaxel to become a reference taxane in breast cancer treatment (Guarneri et al. 2012).

9.3.1.16 Nanoparticles Containing Albumin and Antisense Oligonucleotides

Nanoparticles consisting of human serum albumin (HSA) and containing different antisense oligonucleotides (ASO) have been used for drug delivery to tumors. The preparation process has been optimized regarding the amount of solving agent, stabilization conditions, as well as nanoparticle purification. The glutaraldehyde cross-linking procedure of the particle matrix is a crucial parameter for biodegradability and drug release of the nanoparticles. The drug loading efficiency increases with longer chain length and employment of a phosphorothioate backbone. The resulting nanoparticles can be tested in cell cultures for cytotoxicity and cellular uptake. All cell lines show a significant cellular uptake of HSA nanoparticles. The entrapment of a fluorescent-labeled oligonucleotide within the particle matrix can be used for the detection of the intracellular drug release of the carrier systems. Confocal laser scanning microscopy reveals that nanoparticles cross-linked with low amounts of glutaraldehyde, rapidly degrade intracellularly, leading to a significant accumulation of the ASO in cytosolic compartments of the tumor cells.

9.3.1.17 PEGylated Nanoliposomal Formulation

PEG-coated nanoparticles remain in the tumors and bloodstream longer compared to gelatin nanoparticles. The coating prevents the nanoparticles from removal by the reticuloendothelial system. This property has led to more effective nanoparticles with tumor-targeting properties.

Ceramide, an antimitogenic and proapoptotic sphingolipid, accumulates in cancer tissues and helps to kill cancer cells when patients undergo chemotherapy and radiation. Although the mechanism remains unknown, ceramide is inherently attracted to tumor cells. In vitro tumor cell culture models have shown the potential therapeutic utility of raising the intracellular concentration of ceramide. However, therapeutic use of systemically delivered ceramide is limited by its inherent insolubility in the blood as it is a lipid as well as its toxicity when injected directly into the bloodstream. Packaging ceramide in nanoliposome capsules allows them to travel through the bloodstream without causing toxicity and release the ceramide in the tumor. Systemic intravenous delivery of C6-ceramide (C6) in a PEGylated liposomal formulation significantly limited the growth of solid tumors in a syngeneic BALB/c

mouse tumor model of breast adenocarcinoma (Stover et al. 2005). A pharmacokinetic analysis of systemic liposomal-C6 delivery showed that the PEGylated liposomal formulation follows first-order kinetics in the blood and achieves a steady-state concentration in tumor tissue. Intravenous liposomal-C6 administration was also shown to diminish solid tumor growth in a human xenograft model of breast cancer. In this study in mice, the ceramide bundles targeted and destroyed only breast cancer cells, sparing the surrounding healthy tissue. Together, these results indicate that bioactive ceramide analogs can be incorporated into PEGylated liposomal vehicles for improved solubility, drug delivery, and antineoplastic efficacy. The next step is to explore how additional chemotherapeutic agents could be incorporated into the liposomes for a more lasting effect.

9.3.1.18 Peptide-Linked Nanoparticle Delivery

The coupling chemistry and surface charge effects of peptide labeling in nanoparticle drug delivery strategies are more difficult to control than using folate. Chemical conjugation to peptides reduces colloidal stability, which is a limiting factor in the development of targeting nanoparticles. However, the successful peptide targeting of structural, hormonal, cytokine and endocrine receptors in the delivery of therapeutic and diagnostic radionuclides provides justification for finding methods to synthesize peptide-targeted nanoparticles (Franzen 2011). Although most of the work so far has been done using gold nanoparticles, biological and polymer nanoparticles are more colloidally stable and present enormous opportunities for coupling to peptides. Further studies are needed to develop peptide targeting for nanoparticles to rival the selectivity that has been achieved with the small-molecule folate.

9.3.1.19 Poly-2-Hydroxyethyl Methacrylate Nanoparticles

Poly-2-hydroxyethyl methacrylate nanoparticles can potentially be used for the controlled release of the anticancer drug doxorubicin and reduction of its toxicity (Chouhan and Bajpai 2009). Suspension polymerization of 2-hydroxyethyl methacrylate (HEMA) results in the formation of swellable nanoparticles of defined composition. Release profiles of doxorubicin can be greatly modified by varying the experimental parameters such as percent loading of doxorubicin and concentrations of HEMA, cross-linker, and initiator. Swelling of nanoparticles and the release of doxorubicin increase with the increase in percentage loading of drug. Absorption spectra of doxorubicin do not change following its capture and release form the nanoparticles, indicating that chemical structure of the drug is likely to be unaffected by the procedure.

9.3.1.20 Polypeptide–Doxorubicin-Conjugated Nanoparticles

Artificial recombinant chimeric polypeptides (CPs), produced from genetically altered *E. coli*, have been shown to spontaneously self-assemble into 50-nm

nanoparticles on conjugation with various chemotherapeutics regardless of their water solubility (MacKay et al. 2009). CPs contain a biodegradable polypeptide that is attached to a short Cys-rich segment. Covalent modification of the Cys residues, with a structurally diverse set of chemotherapeutics, leads to spontaneous formation of nanoparticles over a range of CP compositions and molecular weights. Attachment to one of CPs induces characteristics that the drug alone does not possess. Most chemotherapeutics do not dissolve in water, which limits their ability to be taken in by cells, but attachment to a nanoparticle makes the drug soluble. When used to deliver chemotherapeutics to a murine cancer model, CP nanoparticles have a four-fold higher maximum tolerated dose than free drug and induce nearly complete tumor regression after a single dose. After delivering the drug to the tumor, the delivery vehicle breaks down into harmless by-products, markedly decreasing the toxicity for the recipient. This simple as well as inexpensive strategy can promote co-assembly of drugs, imaging agents, and targeting moieties into multifunctional nanomedicines. Since blood vessels supplying tumors are more porous, or leaky, than normal vessels, the nanoformulation can more easily enter and accumulate within tumor cells. This means that higher doses of the drug can be delivered, increasing its anticancer effects while decreasing the side effects associated with systematic chemotherapy.

9.3.1.21 ProtoSphere Nanoparticle Technology

ProtoSphere™ nanoparticle technology (Abraxis Bioscience Inc.), also referred to as nanoparticle albumin-bound or nab™ technology, was used to integrate biocompatible proteins with drugs to create the nanoparticle form of the drug having a size of about 100–200 nm. SPARC (secreted protein acidic and rich in cysteine), a protein overexpressed and secreted by cancer cells, binds albumin to concentrate albumin-bound cytotoxic drugs at the tumor.

The product Abraxane (ABI-007) is a patented albumin-stabilized nanoparticle formulation of paclitaxel (nab-paclitaxel) designed to overcome insolubility problems encountered with paclitaxel. The solvent Cremophor-EL, used previously in formulations of paclitaxel, causes severe hypersensitivity reactions. To reduce the risk of allergic reactions when receiving Taxol, patients must undergo premedication using steroids and antihistamines and be given the drug using slow infusions. The active component (paclitaxel) can be delivered into the body at a 50 % higher dose over 30 min. This contrasts with Taxol infusions, which can take up to 3 h. Because Abraxane is solvent-free, solvent-related toxicities are eliminated, premedication is not required, and administration can occur more rapidly. Abraxane also has a different toxicity profile than solvent-based paclitaxel, including a lower rate of severe neutropenia. In a randomized phase III trial, the response rate of Abraxane was almost twice that of the solvent-containing drug Taxol. Because Abraxane does not contain solvents, higher doses of paclitaxel could be given which may account in part for its increased anticancer activity. In addition, albumin is a protein that normally transports nutrients to cells and has been shown to accumulate in rapidly growing tumors. Therefore, Abraxane's increased effectiveness may also be due to

preferential delivery of albumin-bound paclitaxel to cancer cells. In addition to the standard infusion formulation of Abraxane, oral and pulmonary delivery formulations are also being investigated.

A randomized controlled phase III clinical trial compared the safety and efficacy of 260 mg/m^2 of Abraxane to 175 mg/m^2 of Taxol administered every 3 weeks in patients with metastatic breast cancer (Gradishar et al. 2005). Abraxane was infused over 30 min without steroid pretreatment and at a higher dose than Taxol, which requires steroid therapy and infusion over 3 h Abraxane was found to be superior to Taxol on lesion response rate as well as on tumor progression rate. In 2005, the FDA approved Abraxane for the treatment of metastatic breast cancer. Abraxane also is being evaluated in non-small-cell lung, ovarian, melanoma, and cervical cancers.

9.3.1.22 Zinc Oxide Nanoparticles for Drug Delivery in Cancer

Zinc oxide (ZnO) nanomaterials provide versatile platforms for biomedical applications, and therapeutic intervention and recent studies demonstrate that they hold considerable promise as anticancer agents. Several of these are under development at the experimental, preclinical, and clinical stages. Through a better understanding of the mechanisms of action and cellular consequences resulting from nanoparticles interactions with cells, the inherent toxicity and selectivity of ZnO nanoparticles against cancer may be improved further to make them attractive new anticancer agents (Rasmussen et al. 2010).

9.3.2 Nanoparticles for Targeted Delivery of Anticancer Therapeutics

Nanosystems are emerging that may be very useful for tumor-targeted drug delivery: novel nanoparticles are preprogrammed to alter their structure and properties during the drug delivery process to make them most effective for the different extra- and intracellular delivery steps (Wagner 2007). This is achieved by the incorporation of molecular sensors that are able to respond to physical or biological stimuli, including changes in pH, redox potential, or enzymes. Tumor-targeting principles include systemic passive targeting and active receptor targeting. Physical forces (e.g., electric or magnetic fields, ultrasound, hyperthermia, or light) may contribute to focusing and triggered activation of nanosystems. Biological drugs delivered with programmed nanosystems also include plasmid DNA, siRNA, and other therapeutic nucleic acids.

Drug delivery systems are being developed that attempt to destroy tumors more effectively by using synthesized smart nanoparticles that target and kill cancer cells while sparing healthy cells. Such particles can be injected intravenously into the blood circulation. Each particle, chemically programmed to have an affinity for the cell wall of tumor, can recognize the cancer cell, anchor itself to it, and diffuse inside the

cell. Once inside, the particle disintegrates, causing a nearly instantaneous release of the drug precisely where it is needed. To be effective, the nanoparticles must evade the body's immune system, penetrate into the cancer cells, and discharge the drugs before being recognized by the cancer cells. Advantages of such systems are:

- They can fool cancer cells, which are very good at detecting and rejecting drugs.
- Provide rapid drug delivery at sufficiently high concentration that can overwhelm the cancer cell's resistance mechanisms.
- Reduction of side effects because the cancer cells are targeted selectively, sparing the normal cells.

Another approach is to use a nanoparticle made of a hydrogen and carbon polymer with anticancer drug bound up in its fabric and attached to a substance that targets cancer cells. Following intravenous injection, the polymer would gradually dissolve on reaching the target and gradually release the drug. One limitation of systemic introduction of such nanoparticles, unless properly targeted, is that they may end up in the liver and spleen. This is an unwanted side effect because once the nanoparticles dissolve in those organs, they release toxic levels of chemotherapy in healthy tissues.

9.3.2.1 Canine Parvovirus as a Nanocontainer for Targeted Drug Delivery

The canine parvovirus (CPV) utilizes transferrin receptors (TfRs) for binding and cell entry into canine as well as human cells. TfRs are overexpressed by a variety of tumor cells and are widely being investigated for tumor-targeted drug delivery. To explore the natural tropism of CPV to TfRs for targeting tumor cells, CPV virus like particles (VLPs) produced by expression of the CPV–VP2 capsid protein in a baculovirus expression system were examined for attachment of small molecules and delivery to tumor cells (Singh et al. 2006). Structural modeling suggested that six lysines per VP2 subunit are presumably addressable for bioconjugation on the CPV capsid exterior. Between 45 and 100 of the possible 360 lysines/particle could be routinely derivatized with dye molecules depending on the conjugation conditions. Dye conjugation also demonstrated that the CPV–VLPs could withstand conditions for chemical modification on lysines. Attachment of fluorescent dyes neither impaired binding to the TfRs nor affected internalization of the 26-nm-sized VLPs into several human tumor cell lines. CPV–VLPs therefore exhibit highly favorable characteristics for development as a novel nanomaterial for tumor targeting.

9.3.2.2 Carbon Magnetic Nanoparticles for Targeted Drug Delivery in Cancer

Using dense medium plasma technology, carbon magnetic nanoparticles (CMNPs) have been synthesized at room temperature and atmospheric pressure. Results of X-ray photoelectron spectroscopy, Fourier transform infrared spectroscopy, and

scanning electron microscopy show that these nanoparticles are composed of spherical particles, 40–50 nm in diameter, with iron/iron oxide particles dispersed in a carbon-based host structure (Ma et al. 2004). Thermal gravimetry/differential thermal gravimetry analysis shows these nanoparticles are stable to temperatures as high as 600 °C. The synthesized CMNP were treated by argon plasma, aminated with ethylenediamine, and subsequently activated by generating aldehyde groups on them. Free doxorubicin (DOX) molecules are then immobilized onto the surfaces of activated CMNP particles to form CMNP–DOX conjugates. The in vitro antiproliferative activity of immobilized doxorubicin in the conjugates has been demonstrated in tumor cell cytotoxicity assays. It is suggested that this CMNP–DOX system can be used for targeted drug delivery systems in cancer.

9.3.2.3 Carbon Nanotubes for Targeted Drug Delivery to Cancer Cells

Carbon nanotube (CNTs) with DNA and RNA wrapped around them to make them biocompatible can be targeted toward cancer cells by attaching additional molecules. Such CNT composites, in combination with laser treatment, may be used to destroy the cancer cells. An improved delivery scheme for intracellular tracking and anticancer therapy uses a novel double functionalization of a carbon nanotube delivery system containing antisense oligodeoxynucleotides as a therapeutic gene and CdTe QDs as fluorescent labeling probes via electrostatically layer-by-layer assembling (Jia et al. 2007).

Chemically functionalized SWCNTs have shown promise in tumor-targeted accumulation in mice and exhibit biocompatibility, excretion, and little toxicity. The anticancer drug paclitaxel (PTX) has been conjugated to branched PEG chains on SWCNTs via a cleavable ester bond to obtain a water-soluble SWCNT–PTX conjugate (Liu et al. 2008). SWCNT–PTX is more efficient in suppressing tumor growth than Taxol in a murine 4T1 breast cancer model, owing to prolonged blood circulation and tenfold higher tumor PTX uptake by SWCNT delivery, likely through EPR. Drug molecules carried into the reticuloendothelial system are released from SWCNTs and excreted via biliary pathway without toxic effects on normal organs. Thus, CNT drug delivery is promising for enhancing treatment efficacy and minimizing side effects of cancer therapy by use of low drug doses. Water-dispersed carbon nanohorns, prepared by adsorption of polyethylene glycol–doxorubicin conjugate (PEG–DXR) onto oxidized single-wall carbon nanohorns, have been shown to be effective anticancer drug delivery carriers when administered intratumorally to human NSCLC-bearing mice (Murakami et al. 2008). There was significant retardation of tumor growth associated with prolonged DXR retention in the tumor.

Although considerable further work is required before any new drugs based on CNTs are developed, it is hoped that it will eventually lead to more effective treatments for cancer. However, it is too early to claim whether carbon-based nanomaterials will become clinically viable tools to combat cancer, although there is definitely room for them to complement existing technologies.

9.3.2.4 Cyclosert System for Targeted Delivery of Anticancer Therapeutics

Cyclosert™ (Calando/Insert Therapeutics) is the first nanoparticle drug transport platform to be designed de novo and synthesized specifically to overcome limitations in existing technologies used for the systemic transport of therapeutics to targeted sites within the body. Based on small cyclic repeating molecules of glucose called cyclodextrins, Cyclosert promotes the ability of cytotoxic drugs to inhibit the growth of human cancer cells while reducing toxicity and remaining non-immunogenic at therapeutic doses. In particular, the system is designed to reduce the toxicity of the drugs until they actually reach the targeted tumor cells where the active drug is released in a controlled fashion. Animal studies have shown that the Cyclosert system can safely deliver tubulysin A, a potent, but highly toxic, anti-tumor agent. In vitro studies have shown the tubulysin–Cyclosert conjugate to be effective against multiple human cancer cell lines. The conjugate is stable and 100 times more water soluble than the free drug. Calando is developing CALAA01, a siRNA, for anticancer use using Cyclosert as a delivery system.

IT-101 (Calando) is a de novo designed experimental therapeutic comprised of linear, cyclodextrin (CD)-containing polymer conjugates of CPT that assemble into 40-nm-diameter nanoparticles via polymer–polymer interactions that involve inclusion complex formation between the CPT and the CD. Particle size, near-neutral surface charge, and CPT release rate were specifically designed into IT-101. Cyclosert platform forms nanoscale constructs with hydrodynamic diameters between 30 and 60 nm. This makes Cyclosert-based drugs ideal for effective delivery to solid tumors. Preclinical animal studies show extended circulation times, tumor accumulation, slow release of the CPT, and anticancer efficacy that directly correlate to the properties of the nanoparticle. Release of CPT can disassemble the nanoparticle into individual polymer chains ~10 nm in size that are capable of renal clearance. IT-101 has been evaluated in patients with relapsed or refractory cancer following two cycles of therapy by intravenous infusion. Interim analysis shows that IT-101 is well tolerated, and pancytopenia is the dose-limiting toxicity (Yen et al. 2007). Pharmacokinetic data were favorable and consistent with results from preclinical animal studies. In the patients studied, IT-101 showed longer half-life, lower clearance, and lower volume of distribution than seen in patients treated with other CPT-based drugs. It is in phase II clinical trials.

9.3.2.5 DNA Aptamer–Micelle for Targeted Drug Delivery in Cancer

Design of a self-assembled aptamer–micelle nanostructure has been reported that achieves selective and strong binding of otherwise low-affinity aptamers at physiological conditions (Wu et al. 2009). Specific recognition ability is directly built into the nanostructures. The attachment of a lipid tail onto the end of nucleic acid aptamers provides these unique nanostructures with an internalization pathway. Other merits include extremely low off rate once bound with target cells, rapid

recognition ability with enhanced sensitivity, low critical micelle concentration values, and dual-drug delivery pathways. To prove the potential detection/delivery application of this aptamer–micelle in biological living systems, the authors mimicked a tumor site in the bloodstream by immobilizing tumor cells onto the surface of a flow channel device. Flushing the aptamer–micelles through the channel demonstrated their selective recognition ability under flow circulation in human whole-blood sample. The aptamer–micelles show great dynamic specificity in flow channel systems that mimic drug delivery in the blood system. Therefore, DNA aptamer–micelle assembly has shown high potential for cancer cell recognition and for targeted in vivo drug delivery applications.

9.3.2.6 Fullerenes for Enhancing Tumor Targeting by Antibodies

Although it was previously possible to attach drug molecules directly to antibodies, scientists have not been able to attach more than a handful of drug molecules to an antibody without significantly changing its targeting ability. That happens, in large part, because the chemical bonds that are used to attach the drugs—strong, covalent bonds—tend to block the targeting centers on the antibody's surface. If an antibody is modified with too many covalent bonds, the chemical changes will destroy its ability to recognize the cancer it was intended to attack.

In order to overcome this limitation, a new class of anticancer compounds have been created that contain both tumor-targeting antibodies and nanoparticles called fullerenes (C60), which can be loaded with several molecules of anticancer drugs like Taxol® (Ashcroft et al. 2006). It is possible to load as many as 40 buckyballs into a single skin cancer antibody called ZME-018, which can be used to deliver drugs directly into melanoma tumors. Certain binding sites on the antibody are hydrophobic (water repelling) and attract the hydrophobic fullerenes in large numbers so multiple drugs can be loaded into a single antibody in a spontaneous manner. No covalent bonds are required, so the increased payload does not significantly change the targeting ability of the antibody. The real advantage of fullerene immunotherapy over other targeted therapeutic agents is likely to be the fullerene's potential to carry multiple drug payloads, such as Taxol plus other chemotherapeutic drugs. Cancer cells can become drug resistant, and one can cut down on the possibility of their escaping treatment by attacking them with more than one kind of drug at a time. The first fullerene immunoconjugates have been prepared and characterized as an initial step toward the development of fullerene immunotherapy.

9.3.2.7 Gold Nanoparticles for Targeted Drug Delivery in Cancer

Gold and silica composite nanoparticles have been investigated as nanobullets for cancer. Gold atoms bind to silicon atoms with dangling bonds and serve as seeds for the growth of Au islands. The large electron affinity of gold causes a significant change in the electronic structure of silica resulting in a substantial reduction in the

highest occupied and the lowest unoccupied molecular orbital and the optical gap, thus allowing it to absorb near-infrared radiation. This suggests that a small cluster can have a similar effect in the treatment of cancer as the large-sized nanoshell but with a different mechanism.

The unique chemical properties of colloidal gold make it a promising targeted delivery approach for drugs or genes to specific cells. The physical–chemical properties of colloidal gold permit more than one protein molecule to bind to a single particle of colloidal gold. CytImmune Sciences Inc. has shown that tumor necrosis factor (TNF) can be bound to gold nanocrystals and delivered safely and effectively to tumor-burdened mice and dogs. CytImmune scientists have characterized and modified the colloidal gold (cAu) particles to optimize binding of TNF to the nanocrystals and also the targeting of the particles to the tumor. The therapeutic compounds that CytImmune is developing are new formulations of the TNF-α, which causes the death of tumors but is toxic to healthy organs. Coupling TNF-α to colloidal gold is expected to improve the safety and effectiveness of anticancer therapy. Specifically two drugs are in development: Aurimune-T and AuriTax. Aurimune-T is manufactured by covalently linking molecules of TNF-α and Thiol-derivatized polyethylene glycol (PEG-THIOL) onto the surface of 25-nm colloidal gold. Intravenously administered Aurimune-T rapidly accumulates in solid tumors implanted in mice and shows little to no accumulation in the reticuloendothelial system or in other healthy organs. Coincident with the sequestration of gold is a tenfold accumulation of TNF-α in the tumor when compared to animals treated with native TNF-α. By getting more TNF-α to the tumor, Aurimune-T improves the safety and efficacy of TNF-α treatment since maximal tumor responses were achieved at lower doses of the drug. The second nanoparticle drug, AuriTax, consists of TNF-α, a chemotherapeutic (paclitaxel) and PEG-THIOL, which are bound to the same cAu nanoparticle. Like Aurimune-T, AuriTax delivers tenfold more TNF-α and paclitaxel to the solid tumor when compared to each drug alone. These results support the continued development of the colloidal gold platform for cancer therapy and TNF-α as a tumor-targeting ligand.

Biocompatible and nontoxic PEGylated gold nanoparticles with surface-enhanced Raman scattering have been used for in vivo tumor targeting and detection (Qian et al. 2008). Colloidal gold has been safely used to treat rheumatoid arthritis for 50 years and has recently been found to amplify the efficiency of Raman scattering by 14–15 orders of magnitude. It has been shown that large optical enhancements can be achieved under in vivo conditions for tumor detection in live animals. An important finding is that small-molecule Raman reporters such as organic dyes are not displaced but were stabilized by thiol-modified polyethylene glycols. These PEGylated SERS nanoparticles are considerably brighter than semiconductor QDs with light emission in the near-infrared window. When conjugated to tumor-targeting ligands such as single-chain variable fragment antibodies (ScFv), the conjugated nanoparticles are able to target tumor biomarkers such as EGFRs on human cancer cells and in xenograft tumor models. ScFv peptides bind cancer cells, and the gold particles latch onto tumors after their injection into a mouse. When illuminated with a laser beam, the tumor-bound particles emit a signal that is specific to the dye.

The signal from the dye tags is very bright, and the distinct peaks in the dye signal mean several different probes could be used at the same time. The tags' rich spectroscopic signatures provide the capability of using several probes at once, but that will require more sophisticated computational tools. The authors are developing data processing tools and making them available to the NCI's caBIG (cancer Biomedical Informatics Grid) so that the research community can use them. Compared with QDs, the gold particles are more than 200 times brighter on a particle-to-particle basis, although they are about 60 times larger by volume. Covered with a nontoxic polymer, the gold particles are about 60–80 nm in diameter. That's 150 times smaller than a typical human cell and thousands of times smaller than a human hair. The researchers were able to detect human cancer cells injected into a mouse at a depth of 1–2 cm. That makes the gold particles especially appropriate tools for gathering information about head or neck tumors, which tend to be more accessible. The technology will need further adaptation for use with abdominal or lung cancers deep within the body.

PEGylated gold nanoparticles are decorated with various amounts of human transferrin (Tf) to give a series of Tf-targeted particles with near-constant size and electrokinetic potential. Studies in experimental animals with tumors show that quantitative biodistribution of the nanoparticles 24 h after intravenous injections results in their accumulations in the tumors and other organs independent of Tf (Choi et al. 2010a). However, the nanoparticle localization within a particular organ is influenced by the Tf content. In tumor tissue, the content of targeting ligands significantly influences the number of nanoparticles localized within the cancer cells. In liver tissue, high Tf content leads to small amounts of the nanoparticles residing in hepatocytes, whereas most nanoparticles remain in nonparenchymal cells. These results suggest that targeted nanoparticles can provide greater intracellular delivery of therapeutic agents to the cancer cells within solid tumors than their nontargeted analogs.

Selective transport of gold nanoparticles to the nuclei of cancer cells has been achieved by properly conjugating them with specific peptides (Kang et al. 2010). Localization of gold nanoparticles at the nucleus of a cancer cell damages the DNA resulting in double-strand breaks. Dark-field imaging of live cells in real time revealed that the nuclear targeting of gold nanoparticles specifically induces cytokinesis arrest in cancer cells leading apoptosis.

Prostate tumor-specific epigallocatechin gallate (EGCg) functionalized radioactive gold nanoparticles (AuNPs), when delivered intratumorally, circumvent transport barriers, resulting in targeted delivery of therapeutic payloads. Gold nanoparticles derived from the Au-198 isotope with therapeutic range of 11 mm in tissue have been developed, which is sufficiently long to provide cross-fire effects of a radiation dose delivered to cells within the prostate gland and short enough to minimize the radiation dose to critical tissues near the periphery of the capsule. The formulation of biocompatible 198Au nanoparticles utilizes the redox chemistry of prostate tumor-specific phytochemical EGCg as it converts gold salt into gold nanoparticles and also selectively binds with excellent affinity to Laminin67R receptors, which are overexpressed in prostate tumor cells. Therapeutic studies showed 80 % reduction of tumor

volumes in PC-3 xenograft severe combined immune deficiency (SCID) mice after 28 d demonstrating significant inhibition of tumor growth compared to controls (Shukla et al. 2012). This innovative nanotechnological approach serves as a basis for designing biocompatible target-specific antineoplastic agents. This novel intratumorally injectable 198AuNP-EGCg nanotherapeutic agent may provide significant advances in oncology for use as an effective treatment for prostate and other solid tumors.

9.3.2.8 Magnetic Nanoparticles for Remote-Controlled Drug Delivery to Tumors

Remotely controlled nanoparticles, when pulsed with an electromagnetic field, can release anticancer drugs into tumors (Derfus et al. 2007). The innovation could lead to the improved diagnosis and targeted treatment of cancer. In an earlier work, injectable multifunctional nanoparticles were designed to flow through the bloodstream, home to tumors, and clump together. Clumped particles help in the visualization of tumors by MRI. The system that makes it possible consists of particles that are superparamagnetic, a property that causes them to give off heat when they are exposed to a magnetic field. Tethered to these particles are active molecules, such as anticancer drugs. Exposing the particles to a low-frequency electromagnetic field causes the particles to radiate heat, which melts the tethers and releases the drugs. The waves in this magnetic field have frequencies between 350 and 400 kHz—the same range as radio waves. These waves pass harmlessly through the body and heat only the nanoparticles. The tethers in the system consist of strands of DNA. Two strands of DNA link together through hydrogen bonds that break when heated. In the presence of the magnetic field, heat generated by the nanoparticles breaks these, leaving one strand attached to the particle and allowing the other to float away with its cargo. One advantage of a DNA tether is that its melting point is tunable. Longer strands and differently coded strands require different amounts of heat to break. This heat-sensitive tunability makes it possible for a single particle to simultaneously carry many different types of cargo, each of which can be released at different times or in various combinations by applying different frequencies or durations of electromagnetic pulses. To test the particles, the researchers implanted mice with a tumorlike gel saturated with nanoparticles. They placed the implanted mouse into the well of a cup-shaped electrical coil and activated the magnetic pulse. The results confirm that without the pulse, the tethers remain unbroken. With the pulse, the tethers break and release the drugs into the surrounding tissue. The experiment is a proof of principal demonstrating a safe and effective means of tunable remote activation. However, work remains to be done before such therapies become viable in the clinic.

9.3.2.9 Mesoporous Silica Nanoparticles

MSNs have a considerable potential for drug delivery applications due to their flexibility and high drug load potential. MSNs are biodegradable and mostly eliminated

through renal clearance. Although numerous reports demonstrate sophisticated drug delivery mechanisms in vitro, the therapeutic benefit of these systems for in vivo applications has been uncertain in the past. Recent preclinical data has demonstrated that MSNs are safe and a biocompatible technology platform for targeted drug delivery, passive as well as folate-directed, in cancer. MSNs' medical applicability in vivo has been demonstrated for both drug delivery and diagnostics. Therapeutic efficacy of MSNs has been shown in vivo following oral, local, subcutaneous, or intravenous administration, and MSNs have been approved for clinical trials. Incorporation of multiple therapeutic and diagnostic agents into MSNs is feasible, and this will enable diagnostic-guided therapy. There are still several issues to be considered before MSNs can be used in clinical practice (Rosenholm et al. 2012).

9.3.2.10 Nanobees for Targeted Delivery of Cytolytic Peptide Melittin

The in vivo application of cytolytic peptides for cancer therapeutics is hampered by toxicity, nonspecificity, and degradation. A specific strategy was developed to synthesize a nanoscale delivery vehicle for cytolytic peptides by incorporating the synthetic version of a toxin called melittin that is found in bees into the outer lipid monolayer of a perfluorocarbon (PFC) nanoparticle. The composite structures, called nanobees, are engineered to travel directly to tumor cells without harming any others. They spare the healthy cells but attach to tumor blood vessels, which express a particular protein to which a substance on the nanobees has a chemical affinity. Melittin, which would destroy red blood cells and other normal tissues if it is delivered intravenously, is completely safe when carried on a nanoparticle. Favorable pharmacokinetics of this nanocarrier have been demonstrated, which allow accumulation of melittin in murine tumors in vivo and a dramatic reduction in tumor growth without any apparent signs of toxicity (Soman et al. 2009). Furthermore, direct assays demonstrated that molecularly targeted nanocarriers selectively delivered melittin to multiple tumor targets, including endothelial and cancer cells, through a hemifusion mechanism. In cells, this hemifusion and transfer process did not disrupt the surface membrane but did trigger apoptosis and in animals caused regression of precancerous dysplastic lesions. Collectively, these data suggest that the ability to restrain the wide-spectrum lytic potential of a potent cytolytic peptide in a nanovehicle, combined with the flexibility of passive or active molecular targeting, represents an innovative molecular design for chemotherapy with broad-spectrum cytolytic peptides for the treatment of cancer at multiple stages. So far nanobees have been tested only on mice, with promising results, but what works in mice does not always work in humans. If proven to be effective in humans, this therapy could become widely available in about 5–10 years.

9.3.2.11 Nanobody-Shell Polymeric Micelles for Targeted Drug Delivery

PEG-b-poly[*N*-(2-hydroxypropyl) methacrylamide-lactate] cross-linked thermosensitive biodegradable polymeric micelles suitable for active tumor targeting have

been developed by coupling the anti-EGFR EGa1 nanobody to their surface (Talelli et al. 2011). The micellar conjugates were characterized using SDS-PAGE and gel permeation chromatography. The conjugation was successful as demonstrated by Western blot and dot blot analysis. Rhodamine-labeled EGa1-micelles showed substantially higher binding as well as uptake by EGFR-overexpressing cancer cells than untargeted rhodamine-labeled micelles. No binding was observed of the nanobody micelles to EGFR-negative cells as well as to 14C cells in the presence of an excess of free nanobody. This demonstrates that the binding of the nanobody micelles is by interaction with the EGFR. These polymeric micelles are highly promising systems for active drug targeting.

9.3.2.12 Nanovehicles for Targeted Delivery of Paclitaxel

Nanoparticles have been used to deliver paclitaxel, an antitumor drug, directly to tumors for targeted anticancer treatment. The nanoparticles were loaded with paclitaxel and then mixed with lipids to form nanoparticle-like clusters, which were then coated with a glycosaminoglycan (GAG). The nanovehicle was formed of clusters of loaded nanoparticles then coated with a GAG. When these clusters come into contact with cancerous cells, the paclitaxel is released from the individual nanoparticles directly into the cancerous cell. This targeted release allows the treatment to be focused to the cancerous cells, reducing the negative effects of the chemotherapy treatment.

The ability of the nanovehicles to specifically target the cancerous cells is due to the specific GAG used in the coating of the clusters. The researchers use hyaluronan, a sugar that is recognized by receptors on many different types of cancer cell. When the nanovehicle interacts with the cancer cell, the sugar is recognized by the receptors, triggering a structural change and the subsequent release of the paclitaxel directly into the cell. The release of the paclitaxel directly into the target cell also overcomes the issues associated with the solubility of the drug (Rivkin et al. 2010). The vehicle is similar to a cluster bomb, when the delivery vehicle, comprising of multiple nanoparticles, comes into contact with cancer cells, it releases the chemotherapeutic payload directly into the cell. Peer also mentions that the device can be used to treat a variety of cancers, such as blood, colon, breast, pancreatic, ovarian, and several types of brain cancer. The ability of the nanovehicle to be recognized by the cancer cells means that it increases the effectiveness of the treatment while healthy cells are unaffected by the treatment. Tests on tumor-bearing mice have demonstrated that the nanoclusters are more effective when compared with free paclitaxel and other albumin nanoparticles containing paclitaxel. The nanoclusters also demonstrated a high safety profile and a high level of accumulation within tumors.

The fabrication of the nanovehicles means that the treatment may potentially be safer than alternative current therapies. The nanocluster is formed of the naturally occurring lipid hyaluronan, a lipid that decomposes in the body once the nanoparticles have delivered the paclitaxel. The researchers hope that GAGs may provide vehicles for other chemotherapy drugs, such as taxanes, and may also be used as carriers for other therapeutic applications.

9.3.2.13 Nanocell for Targeted Drug Delivery to Tumor

Simultaneous delivery of chemotherapeutic and antiangiogenic drugs is clearly beneficial, but because chemotherapy is blood borne, shutting down the tumor's blood supply with antiangiogenic drugs may decrease the delivery of drugs designed to kill the tumor cells. A more effective strategy would be to use a delivery vehicle that became concentrated in tumors before the vasculature shuts down and allows the staged release of the two drugs. The delivery of the antiangiogenic factor could lead to a collapse of the vascular network and imprison the vehicle, which would still be carrying its second payload of chemotherapeutic drug, inside the tumor. The subsequent release of the latter drug within the tumor would kill the cancer cells. For example, a composite nanocell can be constructed with a solid biodegradable polymer core surrounded by a lipid membrane in which the outer membrane is loaded with the antiangiogenic drug combretastatin and the inner membrane with the chemotherapy drug doxorubicin. The nanocells are small enough to pass through tumor blood vessels, but they are too large to pass through the pores of normal vessels. Once inside the tumor, the nanocell's outer membrane can disintegrate, releasing the antiangiogenic drug and causing the collapse of the blood vessels feeding the tumor. The collapsed blood vessels trap the nanocell inside the tumor. The nanocell can then slowly release the chemotherapy drug. Although the effect of the sequential delivery of these two drugs on tumor growth is dramatic, these results cannot be quickly translated into therapy for humans. There is a concern that antiangiogenic drugs may promote the spread of tumors to other tissues. Also, in contrast to combretastatin, many antiangiogenic drugs require prolonged tissue exposure to shut down the vasculature and so may not be amenable to this particular approach with a short exposure time.

9.3.2.14 Nanodiamonds for Local Delivery of Chemotherapy at Site of Cancer

Nanodiamonds (NDs) with a diameter of 2–8 nm, physically bound with doxorubicin and sandwiched between a base and thin layer of polymer parylene, enable extended targeted and controlled release at diseased areas in cancer, viral infection, and inflammation (Lam and Ho 2009). A substantial amount of drug can be loaded onto clusters of nanodiamonds, which have a high surface area. Nanodiamonds have many other advantages for drug delivery. They can be functionalized with nearly any type of therapeutic. They can be suspended easily in water, which is important for biomedical applications. They are very scalable and can be produced in large quantities. In control experiments, where the drug was administered without the nanodiamonds, virtually the whole drug was released within 1 day. By adding the drug-laden nanodiamonds to the device, drug release was instantly lengthened to the months-long timescale. The FDA-approved polymer parylene displayed the stable and continuous slow release of drug for at least 1 month due to the powerful

sequestration abilities of the DOX–ND complex. The device also avoids the massive initial release of the drug, which is a disadvantage of conventional therapy.

The nanodiamonds are quite economical and have already been mass-produced as lubrication components for automobiles and for use in electronics. Because the fabrication process is devoid of any destructive steps, the DOX–ND conjugates are unaffected and unaltered. The flexible microfilm device resembles a piece of plastic wrap and can be customized easily into different shapes. It can transform conventional treatment strategies and reduce patients' unnecessary exposure to toxic drugs. The biocompatible and minimally invasive device could be used to deliver chemotherapy drugs locally to sites where malignant tumors have been surgically removed.

9.3.2.15 Nanoimmunoliposome-Based System for Targeted Delivery of siRNA

Low transfection efficiency, poor tissue penetration, and nonspecific immune stimulation by in vivo administered siRNAs have delayed their therapeutic applications. Their potential as anticancer therapeutics hinges on the availability of a vehicle that can be systemically administered, safely and repeatedly, and will deliver the siRNA specifically and efficiently to both primary tumors and metastases. A nanosized immunoliposome-based delivery complex (scL) has been developed that will preferentially target and deliver molecules including plasmid DNA and ASO to tumor cells following systemic administration (Pirollo et al. 2007). This tumor-targeting nanoparticle delivery vehicle can also deliver siRNA to both primary and metastatic disease. The efficiency of this complex has been enhanced by the inclusion of a pH-sensitive histidine–lysine peptide in the complex (scL-HoKC) and by delivery of a modified hybrid (DNA-RNA) anti-HER-2 siRNA molecule. Scanning probe microscopy confirms that this modified complex maintains its nanoscale size. More importantly, this nanoimmunoliposome anti-HER-2 siRNA complex can sensitize human tumor cells to chemotherapeutics, silence the target gene, affect its downstream pathway components in vivo, and significantly inhibit tumor growth in a pancreatic cancer model. This complex has the potential to help translate the potent effects of siRNA into a clinically viable anticancer therapeutic.

9.3.2.16 Nanoparticle-Mediated Targeting of MAPK Signaling Pathway

The MAPK signal transduction cascade is dysregulated in a majority of human tumors, and nanoparticle-mediated targeting of this pathway can optimize cancer chemotherapy. Nanoparticles engineered from a polymer that is chemically conjugated to a selective MAPK inhibitor, PD98059, are taken up by cancer cells through endocytosis and demonstrate sustained release of the active agent, resulting in the inhibition of phosphorylation of downstream extracellular signal regulated kinase (Basu et al. 2009). Modification of the polymer, which is biocompatible as well as

biodegradable and approved by the FDA, leads to a 20-fold increase in drug loading capacity. Nanoparticle-mediated targeting of MAPK has been shown to inhibit the proliferation of melanoma and lung carcinoma cells and induce apoptosis in vitro. Administration of the PD98059-nanoparticles in melanoma-bearing mice inhibits tumor growth and enhances the antitumor efficacy of cisplatin chemotherapy. This study shows the nanoparticle-mediated delivery of signal transduction inhibitors is a potentially effective method of cancer chemotherapy.

9.3.2.17 Nanoparticles for Targeted Antisense Therapy of Cancer

ASO against specific molecular targets (e.g., bcl-2 and Raf-1) are important reagents in cancer biology and therapy. Phosphorothioate modification of the ASO backbone has resulted in an increased stability of ASO in vivo without compromising, in general, their target selectivity. Although the power of antisense technology remains unsurpassed, dose-limiting side effects of modified ASO and inadequate penetration into the tumor tissue have necessitated further improvements in ASO chemistry and delivery systems. Oligonucleotide delivery systems may increase stability of the unmodified or minimally modified ASO in plasma, enhance uptake of ASO by tumor tissue, and offer an improved therapy response. An overview of ASO design and in vivo delivery systems with focus on preclinical validation of a liposomal nanoparticle containing minimally modified raf antisense oligodeoxynucleotide (LErafAON) has been published (Zhang et al. 2009). Intact rafAON (15-mer) is present in plasma and in normal and tumor tissues of athymic mice systemically treated with LErafAON. Raf-1 expression is decreased in normal and tumor tissues of LErafAON-treated mice. Therapeutic benefit of a combination of LErafAON and radiation or an anticancer drug exceeds radiation or drug alone against human prostate, breast, and pancreatic tumors grown in athymic mice. Further improvements in ASO chemistry and nanoparticles are promising avenues in antisense therapy of cancer.

9.3.2.18 Nanoparticles for Delivery of Suicide DNA to Prostate Tumors

A prostate-specific, locally delivered gene therapy has been developed for the targeted killing of prostate cells using C32/DT-A, a degradable polymer nanoparticulate system, to deliver a diphtheria toxin suicide gene (DT-A) driven by a prostate-specific promoter to cells (Peng et al. 2007). These nanoparticles were directly injected to the normal prostate and to prostate tumors in mice. Nearly 50 % of normal prostates showed a significant reduction in size, attributable to cellular apoptosis, whereas injection with naked DT-A-encoding DNA had little effect. A single injection of C32/DT-A nanoparticles triggered apoptosis in 80 % of tumor cells present in the tissue. It is expected that multiple nanoparticle injections would trigger a greater percentage of prostate tumor cells to undergo apoptosis. These results suggest that

local delivery of polymer/DT-A nanoparticles may have application in the treatment of benign prostatic hypertrophy and prostate cancer.

9.3.2.19 Nanoparticles for Targeted Delivery of Concurrent Chemoradiation

The development of chemoradiation—the concurrent administration of chemotherapy and radiotherapy—has led to significant improvements in local tumor control and survival. However, it is limited by its high toxicity. A novel NP therapeutic, ChemoRad NP, has been developed, which can deliver biologically targeted chemoradiation (Wang et al. 2010). This is a biodegradable and biocompatible lipid–polymer hybrid NP that is capable of delivering both chemotherapy and radiotherapy. Using docetaxel, indium^{111} and yttrium^{90} as model drugs, ChemoRad NP was shown to encapsulate chemotherapeutics (up to 9 % of NP weight) and radiotherapeutics (100 mCi of radioisotope per gram of NP) efficiently and deliver both effectively. Targeted delivery of ChemoRad NPs and high therapeutic efficacy of ChemoRad NPs were demonstrated using prostate cancer as a disease model. ChemoRad NP represents a new class of therapeutics that holds great potential to improve cancer treatment.

9.3.2.20 Nanoparticle-Based Therapy Targeted to Cancer Metastases

Early detection of metastases plays an important role in the management of metastatic cancer. In patients with prostate cancer who undergo surgical lymph node resection or biopsy, MRI with lymphotropic superparamagnetic nanoparticles can correctly identify all patients with nodal metastases. This diagnosis is not possible with conventional MRI alone and has implications for the management of men with metastatic prostate cancer, in whom adjuvant androgen deprivation therapy with radiation is the mainstay of management.

Nanoparticle formulations of anticancer drugs may be more effective against cancer metastases. Nanoparticles have the ability to transport complex molecular cargoes to the major sites of metastasis, such as the lungs, liver, and lymph nodes, as well as targeting to specific cell populations within these organs (Schroeder et al. 2012). Oral administration of alpha-TEA formulated in liposome or biodegradable poly(D,L-lactide-co-glycolide) nanoparticle has been shown to significantly reduce tumor burden in a mammary cancer mouse model (Wang et al. 2007). Both formulations reduced lymph node and lung micrometastatic tumor foci, but nanoparticle formulation was more effective in reducing metastases. Tumor targeting with nanoparticles facilitates systemic delivery of immunomodulatory cytokine genes to remote sites of cancer metastasis. Targeted delivery and localized expression of the intravenously administered nanoparticles bearing the gene encoding granulocyte–macrophage colony-stimulating factor were confirmed in a patient with metastatic cancer, as was the recruitment of significant tumor-infiltrating lymphocytes (Gordon et al. 2008).

9.3.2.21 Nanoparticle-Mediated Delivery of Multiple Anticancer Agents

Concomitant delivery of the multiple anticancer agents by nanocarriers is expected to enhance the synergistic effects for the following reasons (Mi et al. 2012):

- Nanocarriers can encapsulate large quantities of the therapeutic agents.
- Cellular uptake of the nanocarriers is efficient due to internalization by endocytosis.
- Nanocarriers 100–200 nm in diameter with a surface coating such as PEG or vitamin E TPGS (tocopheryl polyethylene glycol succinate) can escape elimination by macrophages and enable sustained delivery.
- Nanocarriers may provide high oral bioavailability of the formulated agents.

9.3.2.22 Nanostructured Hyaluronic Acid for Targeted Drug Delivery in Cancer

Active targeting of bioactive molecules by nanoparticulate delivery systems that include hyaluronic acid (HA) in their structures is an attractive approach to drug delivery because HA is biocompatible, nontoxic, and noninflammatory. To make HA useful as an intravenous targeting carrier, strategies have to be devised to reduce its clearance from the blood, suppress its uptake by liver and spleen, and provide tumor-triggered mechanisms of release of an active drug from the HA carrier (Ossipov 2010).

HA nanoparticles (HA-NPs), which are formed by the self-assembly of hydrophobically modified HA derivatives, have been tested for their physicochemical characteristics and fates in tumor-bearing mice after systemic administration (Choi et al. 2010b). Irrespective of the particle size, significant amounts of HA-NPs are circulated for 2 days in the bloodstream and selectively accumulated into the tumor. The smaller HA-NPs were able to reach the tumor more effectively than larger HA-NPs. The concentration of HA-NPs in the tumor site was dramatically reduced when mice were pretreated with an excess of free HA. These results indicate that HA-NPs can accumulate into the tumor site by a combination of passive and active targeting mechanisms.

9.3.2.23 Perfluorocarbon Emulsion for Targeted Chemotherapeutic Delivery

Kereos Inc.'s emulsion particles consist of a PFC core surrounded by a lipid monolayer, which stabilizes the particle in addition to providing a virtually unlimited number of anchoring sites for targeting ligands and payload molecules. The result is an oil-in-water emulsion of particles with an average size of approximately 250 nm, referred to as "targeted nanoparticles." Delivered by injection, this approach offers the following advantages:

- High molecular specificity of MAbs, small-molecule ligands, and other targeting ligands for disease biomarkers translates directly into high specificity of the emulsion particles for disease sites.

- Although only 10–100 targeting ligand molecules are needed to direct and securely bind an individual emulsion particle to the disease site, each particle can carry 100,000 or more payload molecules. This "signal amplification" opens up opportunities not otherwise possible.
- Both in terms of size and composition, the emulsion particles are designed to be both safe and effective and to avoid potential problems with distribution, metabolism, or excretion.

9.3.2.24 Polymer Nanoparticles for Targeted Drug Delivery in Cancer

Cerulean Nanopharmaceuticals' CRLX101 (formerly IT-101), a cyclodextrin polymer-based nanoparticle containing CPT, is in phase IIa clinical development for the treatment of cancer. PET data from ^{64}Cu-labeled CRLX101 to quantify the in vivo biodistribution in mice bearing tumors shows that ~8 % of the injected dose is rapidly cleared as a low molecular weight fraction through the kidneys, and the remaining material circulates in plasma with a terminal half-life of 13.3 h (Schluep et al. 2009). A 3-compartment model is used to determine vascular permeability and nanoparticle retention in tumors and is able to accurately represent the experimental data. The calculated tumor vascular permeability indicates that the majority of nanoparticles stay intact in circulation and do not disassemble into individual polymer strands. A key assumption to modeling the tumor dynamics is that there is a sink for the nanoparticles within the tumor. Histological measurements using confocal microscopy show that CRLX101 localizes within tumor cells and provides the sink in the tumor for the nanoparticles.

Several mechanisms have been proposed to explain nanoparticle retention in tumors:

1. Dextran-coated iron oxide nanoparticles accumulate in the interstitial fluid and are taken up by tumor vascular endothelial cells, which observed mostly in areas of neovascularization whereas intracellular concentrations are highest in tumor cells.
2. Long-circulating liposomes accumulate predominantly in tumor stroma, either in the extracellular space or in tumor-associated macrophages in a breast cancer tumor model. A Her2-targeted version of the same liposomes achieves the same over all tumor concentration, but more internalization by cancer cells through endocytosis is observed.
3. Cyclodextrin-based polymer (CDP) conjugates have been shown to be avidly taken up by cancer cells. This result may be a function of the unique surface characteristics of CDP nanoparticles, which contain hydrophobic pockets within the cyclodextrin molecules that have been shown to interact with lipid rafts of cell membranes.

Scientists at the MIT-Harvard Center for Cancer Nanotechnology Excellence (Cambridge, MA) have studied the effects of altering nanoparticle polymer composition, drug loading, and solvents on the ability of the resulting nanoparticles to target and deliver drugs to tumors. As a targeting agent for all the polymer

nanoparticles studied, they used a molecule that recognizes the prostate-specific membrane antigen (PSMA). The aim of this study was to develop formulation parameters that would control the size of the resulting polymer nanoparticles, which the investigators believe play a major role in optimizing tumor targeting. Nanoparticles were prepared from a biocompatible material. Experimenting with a variety of polymer concentrations and solvent mixtures, they found that they could systematically control the size of the resulting polymers. The results were so consistent that the investigators believe that they may have developed a broadly applicable approach to reproducibly tuning the size of polymer nanoparticles during their formulation. In a final experiment, the researchers added the targeting agent to their optimized nanoparticles. The targeted nanoparticles were able to significantly increase drug delivery to human prostate tumors growing in mice.

9.3.2.25 Polymersomes for Targeted Cancer Drug Delivery

Polymersomes, hollow-shell nanoparticles, have unique properties of that allow them to deliver two distinct drugs, paclitaxel and doxorubicin, directly to tumors implanted in mice. Loading, delivery, and cytosolic uptake of drug mixtures from degradable polymersomes can exploit both the thick membrane of these block copolymer vesicles and their aqueous lumen as well as pH-triggered release within endolysosomes. Drug-delivering polymersomes break down in the acidic environment of the cancer cells resulting in targeted release of these drugs within tumor cells. While cell membranes and liposomes (vesicles often used for drug delivery) are created from a double layer of fatty molecules called phospholipids, a polymersome is comprised of two layers of synthetic polymers. The individual polymers are degradable and considerably larger than individual phospholipids but have many of the same chemical features. The large polymers making up the shell allow paclitaxel, which is water insoluble, to embed within the shell. Doxorubicin, which is water soluble, stays within the interior of the polymersome until it degrades. The polymersome and drug combination is self-assembling; the structure spontaneously forms when all of the components are suitably mixed together. Recent studies have shown that cocktails of paclitaxel and doxorubicin lead to better tumor regression than either drug alone, but previously there was no carrier system that could carry both drugs as efficiently to a tumor. Polymersomes get around those limitations.

Another approach is by assembling diverse bioactive agents, such as DNA, proteins, and drug molecules, into core-shell multifunctional PNPs that can be internalized in human breast cancer cells (Bertin et al. 2006). Using ring-opening metathesis polymerization, block copolymers containing small-molecule drug segments (>50 % w/w) and tosylated hexaethylene glycol segments were prepared and assembled into PNPs that allowed for the surface conjugation of single-stranded DNA sequences and/or tumor-targeting antibodies. The resulting antibody-functionalized particles were readily uptaken by breast cancer cells that overexpressed the corresponding antigens.

9.3.2.26 Quantum Dots and Quantum Rods for Targeted Drug Delivery in Cancer

A single-particle QD conjugated with a tumor-targeting MAb (anti-HER2) has been tracked in tumors of live mice (Tada et al. 2007). The researchers used a dorsal skinfold chamber and a high-speed confocal microscope with a high-sensitivity camera to track the antibody-labeled QDs and made 30-frame-per-second movies of these nanoparticles (NPs) as they traveled through the bloodstream. The HER2 MAb binds to a protein found on the surface of certain breast and other tumors. This was injected, conjugated to the QDs, into mice with HER2-overexpressing breast cancer to analyze the molecular processes of its mechanistic delivery to the tumor. The investigators identified six distinct "stop-and-go" steps in the process involved in the antibody-labeled QDs traveling from the injection site to the cell where they bind HER2: within a blood vessel in the circulation, during extravasation, in the extracellular region, binding HER2 on the cell membrane, moving into the perinuclear region, and within the perinuclear region. The image analysis of the delivery processes of single particles in vivo thus provides valuable information on antibody-conjugated therapeutic nanoparticles, which will be useful in increasing therapeutic efficacy.

Water-soluble CdSe/CdS/ZnS quantum rods (QRs) have been developed as targeted probes for imaging cancer cell lines using two-photon fluorescence imaging. The researchers first developed a new method of creating QRs that would remain well dispersed in water and then refined the technique to allow the attachment of targeting molecules (in this case, transferrin, which binds to a receptor that is overexpressed in many types of cancer cells) to the QR surface. QRs, similar to the spherical QDs, fluoresce and can be made to fluoresce in a range of colors. However, since QRs have larger dimensions than QDs, they are easier to excite with incoming light than QDs. This research showed that the QRs were only taken up by targeted transferrin-positive cells and accumulated within these cells, being easily visible using low-intensity near-infrared light, which helps to protect cell integrity. If future research can further our understanding of QDs and QRs following these studies, it is hoped that we could then improve the ability of NPs to deliver drugs specifically to tumors, thus resulting in improved cancer diagnostics and therapeutics.

9.3.2.27 Remote-Controlled Drug Delivery from Magnetic Nanocrystals

Combination of magnetic nanocrystals with ability to exhibit hyperthermic effects when placed in an oscillating magnetic field and MSNs that can contain and release drug cargos could provide a unique drug delivery system for cancer. A nanosystem incorporating zinc-doped iron oxide nanocrystals within a mesoporous silica framework has been surface-modified with pseudorotaxanes (Thomas et al. 2010). Upon application of an AC magnetic field, the nanocrystals generate local internal heating, causing the molecular machines to disassemble and allowing the drug cargos to be released. Breast cancer cell (MDA-MB-231) death was achieved in vitro when

doxorubicin-loaded particles were exposed to an AC field. This material has potential as a noninvasive, externally controlled drug delivery system with cancer-killing properties.

9.3.2.28 Targeted Delivery of Nanoparticulate Drugs into Lymphatic System

The lymphatic system plays a major role in the defense cancer and is one of the main pathways for the metastasis of tumors. The regional lymph nodes, when invaded by cancer cells, act as reservoirs from where these cells spread to other parts of the body. The lymphatic system is not easily accessible by conventional intravenous infusion of chemotherapeutics, thus limiting the amount of drug that reaches lymphatic tissues including lymph node metastases. The lymphatics, however, can be exploited as a route for drug delivery as these channels can transport certain lipophilic compounds and chemotherapeutics.

Nanoparticles can be effectively taken up into lymphatics as well as retained in lymph nodes for several days, and without using any specific targeting ligand, they are internalized exclusively by nodal resident dendritic cells (DCs) and other antigen-presenting cells. Animal studies have demonstrated that nanoparticles made of natural or synthetic polymers and liposomal carriers have higher accumulation in the lymph nodes and surrounding lymphatics compared to conventional intravenous therapies (Xie et al. 2009). In vivo studies have shown that up to 40–50 % of resident lymph node DCs internalize nanoparticles, further supporting the feasibility of this delivery strategy. Bioavailability and biodistribution can be controlled easily by varying the size of nanoparticles. Biodegradable nanoparticles of 20–45 nm have shown the potential for immunotherapeutic applications that specifically target DCs in lymph nodes, e.g., targeted delivery of immunomodulating formulations and vaccines. This can diminish toxicity of highly toxic active drugs.

9.3.2.29 Targeted Drug Delivery with Nanoparticle–Aptamer Bioconjugates

Nucleic acid ligands (aptamers) are potentially well suited for the therapeutic targeting of drug-encapsulated controlled release polymer particles in a cell- or tissue-specific manner. Scientists at the Massachusetts Institute of Technology (Cambridge, MA) have synthesized poly(lactic acid)-block-polyethylene glycol (PLA-PEG) copolymer with a terminal carboxylic acid functional group (PLA-PEG-COOH) and encapsulated rhodamine-labeled dextran (as a model drug) within PLA-PEG-COOH nanoparticles (Farokhzad et al. 2004). These nanoparticles have the following desirable characteristics:

- Negative surface charge, which may minimize nonspecific interaction with the negatively charged nucleic acid aptamers

- Carboxylic acid groups on the particle surface for potential modification and covalent conjugation to amine-modified aptamers
- Presence of PEG on particle surface, which enhances circulating half-life while contributing to decreased uptake in nontargeted cells

Nanoparticle–aptamer bioconjugates were generated with RNA aptamers that bind to the PSMA, a well-known prostate cancer tumor marker that is overexpressed on prostate acinar epithelial cells. These bioconjugates could efficiently target and get taken up by the prostate epithelial cells, which express the PSMA protein. The uptake of these particles was not enhanced in cells that do not express the PSMA protein. This represents the first report of targeted drug delivery with nanoparticle–aptamer bioconjugates. Numerous investigators have used aptamers as replacements for antibodies in various therapeutic and diagnostic applications. They can be programmed to release therapeutically useful molecules in response to a programmed molecular signal.

9.3.2.30 Use of T Cells for Delivery of Gold Nanoparticles to Tumors

Gold nanoparticles (AuNPs) are injected intravenously and are allowed to accumulate within the tumor via the EPR effect. Although reliance on the EPR effect for tumor targeting has proven adequate for vascularized tumors in small animal models, the efficiency and specificity of tumor delivery in vivo, particularly in tumors with poor blood supply, may not be adequate. Human T cells, loaded with 45-nm gold colloid nanoparticles, can be used as cellular delivery vehicles for AuNP transport into tumors, without affecting viability or function (e.g., migration and cytokine production). Using a human tumor xenograft mouse model, it was demonstrated that AuNP-loaded T cells retain their capacity to migrate to tumor sites in vivo (Kennedy et al. 2011). In addition, the efficiency of AuNP delivery to tumors in vivo is increased by more than fourfold compared to injection of free PEGylated AuNPs and the use of the T-cell delivery system also dramatically alters the overall nanoparticle biodistribution. Thus, the use of T-cell chaperones for AuNP delivery could enhance the efficacy of nanoparticle-based therapies and imaging applications by increasing AuNP tumor accumulation. This could also be used for thermal destruction of tumor by application of near infrared (NIR) laser.

9.3.3 Dendrimers for Anticancer Drug Delivery

Earlier studies of dendrimers in drug delivery systems focused on their use for encapsulating drug molecules. However, it was difficult to control the release of the drug. One solution to this problem involves the use of dendrimers with pH-sensitive hydrophobic acetal groups on the dendrimer periphery. Loss of acetal group at mildly acidic pH triggers the disruption of micelles and release of the drug. Another

approach is to attach the drug to the periphery of the dendrimer so that the release of the drug can be controlled by incorporating a degradable linkage between the drug and the dendrimer. Dendrimers have been used to facilitate boron neutron capture therapy (BNCT) as well as photodynamic therapy (PDT) of cancer.

Developments in polymer and dendrimer chemistry have provided a new class of molecules called “dendronized polymers,” i.e., linear polymers that bear dendrons at each repeat unit. Their behavior differs from that of linear polymers and provides drug delivery advantages because of their longer circulation time and numerous possibilities for peripheral attachments of drugs.

New developments in polymer and dendrimer chemistry have provided a new class of molecules called “dendronized polymer,” i.e., linear polymer that bear dendrons at each repeat unit. Their behavior differs from that of linear polymers and provides drug delivery advantages because of their longer circulation time and numerous possibilities peripheral attachments of drugs. Modified PAMAM dendritic polymers <5 nm in diameter have been used as drug carriers. They are conjugated to folic acid as a targeting agent and then coupled to methotrexate and injected intravenously into animals bearing tumor that overexpress the folate receptor. Folate molecules bind to receptors on tumor cell membranes and facilitate the transport of methotrexate to inside of the tumor cell.

Doxorubicin (DOX) has been conjugated to a biodegradable dendrimer with optimized blood circulation time through size and molecular architecture, drug loading through multiple attachment sites, solubility through PEGylation, and drug release through the use of pH-sensitive hydrazone linkages. Dendrimer–DOX is >10 times less toxic than free DOX toward colon carcinoma cells in culture. Upon intravenous administration to tumor-bearing mice, tumor uptake of dendrimer–DOX is ninefold higher than intravenous free DOX and caused complete tumor. The antitumor effect of dendrimer–DOX is similar to that of an equimolar dose of liposomal DOX (Doxil). The remarkable antitumor activity of dendrimer–DOX results from the ability of the dendrimer to favorably modulate the pharmacokinetics of attached DOX.

9.3.3.1 Application of Dendrimers in Boron Neutron Capture Therapy

BNCT offers a potential method for localized destruction of tumor cells. The technology is based on the nuclear reaction between thermal neutrons and boron-10 (10B) to yield alpha particles and lithium-7 nuclei. The destructive effect of this reaction is limited to a range of about the diameter of a single cell. In order for BNCT to be effective in cancer therapy, there must be selective delivery of an adequate concentration of 10B to tumors. Various types of antibodies as well as epidermal growth factor have been utilized to investigate receptor-mediated boron delivery; however, in vivo studies have demonstrated only a small percentage of the total administered dose actually accumulates in tumors while high concentrations end up in the liver.

In normal as well as cancer cells, the low molecular weight vitamin, folic acid, is required for a number of enzymatic pathways. Cell membrane receptors mediating endocytic transport of folic acid into cells are expressed in elevated levels in a variety of human tumors. Folic acid conjugates with macromolecules such as toxins, enzymes, antibodies, genes, and liposomes have been shown to be internalized into tumor cells overexpressing folate receptors. These strategies have been employed to enhance the effect of BNCT. The use of dendrimers as boron carriers for antibody conjugation is based on their well-defined structure and multivalency.

Boronated PAMAM dendrimers have been designed to target the epidermal growth factor receptor, a cell surface receptor that is frequently overexpressed in brain tumor cells.

Preclinical evaluation has been described of a multipurpose STARBURST PAMAM (polyamidoamine) dendrimer prototype (Dendritic Nanotechnologies Inc.) that exhibits properties suitable for use as (1) targeted, diagnostic MRI/NIR (near-IR) contrast agents and/or (2) for controlled delivery of cancer therapies (Tomalia et al. 2007). The lead candidate is 1,4-diaminobutane, a dendritic nanostructure ~5 nm in diameter, which was selected on the basis of a very favorable biocompatibility profile on in vitro studies, i.e., benign and non-immunogenic. The expectation is that it will exhibit desirable mammalian kidney excretion properties and demonstrated targeting features.

9.3.3.2 Application of Dendrimers in Photodynamic Therapy

PDT uses light-activated drugs called photosensitizers to treat a range of diseases characterized by rapidly growing tissue, including the formation of abnormal blood vessels, such as cancer and age-related macular degeneration. The more traditional name for this therapy is photoradiation therapy. Treatment with PDT consists of a two-step process that starts with administration of the drug, or photosensitizer, by intravenous injection. Once the drug enters the bloodstream, it attaches itself to low-density lipoproteins already circulating. As cells undergoing rapid growth require an above-average supply of lipoproteins, the drug reaches these types of cells more quickly and in higher concentrations. Once the necessary level of concentration is attained, the second step is to activate the drug with a specific dose of light of a particular wavelength. This causes the conversion of normal oxygen found in tissue to a highly energized form called singlet oxygen, which in turn, disrupts normal cellular functions. Neither the drug nor the light exerts any effect until combined.

Numerous studies have used liposomes, oils, and polymeric micelles as encapsulation methods, with some success. However, all of these techniques suffer from one unpleasant side effect: after controlled release and photosensitization, the drug is free to circulate the body, accumulating in the eyes and skin. This leads to phototoxic side effects, rendering the patient highly sensitive to light. A further disadvantage is that liposomes can be engulfed and destroyed by cells of the reticuloendothelial

system. Such problems have limited the emerging field of PDT, but combination of this technique with nanotechnology is promising.

The possibility of improving dendrimers through appropriate functionalization of their periphery makes them promising carriers of PDT. The use of 5-aminolevulinic acid (ALA) is one approach to PDT based on dendrimers. ALA is a natural precursor of the photosensitizer protoporphyrin IX (PIX), and its administration increases the cellular concentrations of PIX. Cellular uptake of the dendrimer occurs through endocytic routes predominantly via a macropinocytosis pathway. A dendrimer conjugate, which incorporated 18 ALA residues attached via ester linkages to a multipotent aromatic core, has been investigated (Battah et al. 2007). The ability of the dendrimer to deliver and release 5-ALA intracellularly for metabolism to the photosensitizer, PIX, was studied in the transformed PAM 212 murine keratinocyte and A431 human epidermoid carcinoma cell lines. The macromolecular dendritic derivatives were shown to be capable of delivering 5-ALA efficiently to cells for sustained porphyrin synthesis.

Another approach to deep tissue penetration is based on two-photon excitation with near-infrared lasers. Multivalent aspects of dendrimer scaffold can be used to conjugate several 2-photon absorbing chromophores to the porphyrin core. Such a system can generate singlet oxygen efficiently on light irradiation at 780-nm wavelength.

9.3.3.3 Dendrimer-Based Synthetic Vector for Targeted Cancer Gene Therapy

A synthetic vector system based on polypropylenimine dendrimers has the desired properties of a systemic delivery vehicle and mediates efficient transgene expression in tumors after intravenous administration. Specifically, the systemic injection of dendrimer nanoparticles containing a TNF-α expression plasmid regulated by telomerase gene promoters (hTR and hTERT) leads to transgene expression, regression of remote xenograft murine tumors, and long-term survival of up to 100 % of the animals. The combination of pharmacologically active synthetic transfection agent and transcriptionally targeted antitumor gene creates an efficacious gene medicine for the systemic treatment of experimental solid tumors. The promising results of such experiments could make it possible to treat inaccessible tumors in humans using gene therapy in the future. This new treatment can selectively target cancer cells, without causing damage to surrounding healthy cells.

9.3.3.4 Poly-L-Lysine Dendrimer as Antiangiogenic Agent

Poly-L-lysine (PLL) sixth-generation (G6) dendrimer molecules exhibit systemic antiangiogenic activity that could lead to arrest of growth of solid tumors. Intravenous administration of the PLL–dendrimer molecules into C57BL/6 mice inhibits vascularization of tumors grown within dorsal skinfold window chambers as

demonstrated by intravital microscopy (Al-Jamal et al. 2010). The in vivo toxicological profile of the PLL–dendrimer molecules shows that it is safe at the dose regimen studied. The antiangiogenic activity of the PLL dendrimer is further shown to be associated with significant suppression of B16F10 solid tumor volume and delayed tumor growth. Enhanced apoptosis/necrosis within tumors of PLL–dendrimer-treated animals only and reduction in the number of CD31-positive cells is observed in comparison to protamine treatment. This study suggests that PLL–dendrimer molecules can exhibit a systemic antiangiogenic activity that may be used for therapy of solid tumors and in combination with their capacity to carry other therapeutic or diagnostic agents may potentially offer capabilities combining diagnosis with therapy.

9.3.4 RNA Nanotechnology for Delivery of Cancer Therapeutics

RNA has immense promise as a therapeutic agent against cancer, but the problem has been to have an efficient system to bring multiple therapeutic agents directly into specific cancer cells where they can perform different tasks. The 25-nm RNA nanoparticles enable repeated long-term administration and avoid the problems of short retention time of small molecules and the difficulties in the delivery of particles larger than 100 nm. Nanoparticles, which are assembled from three short pieces of RNA and resemble miniature triangles, possess both the right size to gain entry into cells and also the right structure to carry other therapeutic strands of RNA inside with them, where they are able to halt viral growth or cancer's progress. RNA molecules come in many variant forms, and the one mimicked from the phi29 virus—called pRNA—also can be linked to other types of RNA to form longer, hybrid strands with properties that could be assigned. Incubation of cancer with the pRNA dimer, one subunit of which harbored the receptor-binding moiety and the other harboring the gene-silencing molecule, resulted in their binding and entry into the cells and subsequent silencing of anti/proapoptotic genes. The chimeric pRNA complex was found to be processed into functional double-stranded siRNA by Dicer (RNA-specific endonuclease). Animal studies have confirmed the suppression of tumorigenicity of cancer cells by ex vivo delivery.

RNA nanotechnology has been used to engineer both therapeutic siRNA and a receptor-binding RNA aptamer into individual pRNAs of phi29's motor. The RNA building block harboring siRNA or other therapeutic molecules is subsequently incorporated in a trimer through the interaction of engineered right and left interlocking RNA loops. The incubation of the protein-free nanoscale particles containing the receptor-binding aptamer or other ligands results in the binding and co-entry of the trivalent therapeutic particles into cells, which can modulate the apoptosis of cancer cells as shown in animal studies. The use of such antigenicity-free 20–40-nm particles holds promise for the repeated long-term treatment of cancer and other chronic diseases.

9.3.4.1 Delivery of siRNAs for Cancer

Targeted delivery of siRNAs is considered to be safer and more effective therapeutics for oncology applications. Although macromolecules accumulate nonspecifically in tumors through the EPR effect, previous studies using nanoparticles to deliver siRNA demonstrated that attachment of cell-specific targeting ligands to the surface of nanoparticles leads to enhanced potency relative to nontargeted formulations. Although both nontargeted and transferrin-targeted siRNA nanoparticles exhibit similar biodistribution and tumor localization by PET, transferrin-targeted siRNA nanoparticles reduce tumor luciferase activity by ~50 % relative to nontargeted siRNA nanoparticles 1 day after injection (Bartlett et al. 2007). Compartmental modeling is used to show that the primary advantage of targeted nanoparticles is associated with processes involved in cellular uptake in tumor cells rather than overall tumor localization. Optimization of internalization may, therefore, be a key to the development of effective nanoparticle-based targeted siRNA therapeutics.

9.3.5 Tumor Priming for Improving Delivery of Nanomedicines to Solid Tumors

Effectiveness of nanomedicines in cancer therapy is limited in part by inadequate delivery and transport in tumor interstitium. Tumor priming to overcome these limitations includes measures for extravasation and interstitial transport (Wang et al. 2011a, b). Experimental approaches to improve delivery and transport of nanomedicines in solid tumors include tumor vasculature normalization, interstitial fluid pressure modulation, enzymatic extracellular matrix degradation, and apoptosis-inducing tumor priming technology, which is exemplified by enhancement of delivery and efficacy of liposomal doxorubicin by paclitaxel.

9.4 Nanotechnology-Based Cancer Therapy

9.4.1 Devices for Nanotechnology-Based Cancer Therapy

9.4.1.1 Convection-Enhanced Delivery with Nanoliposomal CPT-11

Combination of convection-enhanced delivery (CED) with a novel, highly stable nanoparticle/liposome containing CPT-11 (nanoliposomal CPT-11) is a potential dual-drug delivery strategy for brain tumor treatment. Following CED in rat brains, tissue retention of nanoliposomal CPT-11 was shown to be greatly prolonged, with >20 % injected dose remaining at 12 days (Noble et al. 2006). In contrast, CED of free CPT-11 resulted in rapid drug clearance. At equivalent CED doses,

nanoliposomal CPT-11 increased area under the time–concentration curve by 25-fold and tissue t1/2 by 22-fold over free CPT-11; CED in intracranial U87 glioma xenografts showed even longer tumor retention. Plasma levels were undetectable following CED of nanoliposomal CPT-11. Importantly, prolonged exposure to nanoliposomal CPT-11 resulted in no measurable CNS toxicity at any dose tested, whereas CED of free CPT-11 induced severe CNS toxicity. In the intracranial U87 glioma xenograft model, a single CED infusion of nanoliposomal CPT-11 resulted in significantly improved median survival compared with CED of control liposomes. The study concluded that CED of nanoliposomal CPT-11 greatly prolonged tissue residence while also substantially reducing toxicity, resulting in a highly effective treatment strategy in preclinical brain tumor models.

9.4.1.2 Nanoengineered Silicon for Brachytherapy

BrachySil™ (^{32}P BioSilicon, pSivida Corporation) is a nanoparticle in which the isotope 32-phosphorus is immobilized. It demonstrates a very high degree of isotope retention following injection into the liver, thus reducing the risk of soluble radioactive material affecting healthy hepatic tissue or entering the circulation and causing systemic toxicity. Unlike titanium seeds, which remain forever in the body, phosphorus seeds degrade over time and enable repetition of treatment if necessary. Other treatments for primary liver cancer include a variety of embolization and radio-frequency ablation techniques. BrachySil offers a more versatile and safer product for the treatment of such tumors. The procedure is undertaken without surgery under local anesthetic, and patients can be discharged the following day. A phase IIa trial in primary liver cancer has shown that it is safe and effective in tumor regression with increased efficacy. An efficacy/safety study for the treatment of pancreatic cancer, which was completed in 2008, showed that the treatment was well tolerated with disease control in 82 % of patients and an overall median survival of 309 days.

9.4.2 Anticancer Effect of Nanoparticles

9.4.2.1 Antiangiogenic Therapy Using Nanoparticles

Integrin-targeted nanoparticles can be used for site-specific delivery of a therapeutic payload. Selective targeting of upregulated $\alpha_v\beta_3$ and Flk-1 on the neovasculature of tumors is a novel antiangiogenesis strategy for treating a wide variety of solid tumors.

The mechanism of inhibition of the function of proangiogenic heparin-binding growth factors (HB-GFs), such as vascular endothelial growth factor 165 (VEGF165) and basic fibroblast growth factor (bFGF) by gold nanoparticles (AuNPs), has been investigated (Arvizo et al. 2011). It was shown that a naked GNP surface is required,

and core size plays an important role to inhibit the function of HB-GFs and subsequent intracellular signaling events. The authors also demonstrated that the inhibitory effect of GNPs is due to the change in HB-GFs conformation/configuration (denaturation) by the NPs, whereas the conformations of non-HB-GFs remain unaffected. This study will help structure-based design of therapeutic NPs.

9.4.2.2 Cytotoxic Effects of Cancer Nanoparticles

Nanoparticles may have a direct cytotoxic effect on cancer cells by various mechanisms. DNA degradation and anticancer activity of copper nanoparticles of 4–5-nm size have been reported, e.g., dose-dependent degradation of isolated DNA molecules by copper nanoparticles through generation of singlet oxygen. Singlet oxygen scavengers such as sodium azide and tris [hydroxyl methyl] amino methane were able to prevent the DNA degradation (Jose et al. 2011). Additionally, it was observed that the copper nanoparticles are able to exert cytotoxic effect toward U937 and Hela cells of human histiocytic lymphoma and human cervical cancer origins, respectively, by inducing apoptosis.

9.4.2.3 Gold Nanoparticles for Inhibiting Tumor Growth

A study has demonstrated that unmodified gold nanoparticles (AuNPs) inhibit the proliferation of cancer cells in a size- and concentration-dependent manner by abrogating MAPK signaling (Arvizo et al. 2013). In addition, these AuNPs reverse epithelial–mesenchymal transition (EMT) in cancer cells by reducing secretion of a number of proteins involved in EMT, upregulating E-cadherin, and downregulating Snail, N-cadherin, and vimentin. Inhibition of MAPK signaling and reversal of EMT upon AuNP treatment inhibit tumor growth and metastasis in two separate orthotopic models of ovarian cancer. Western blot analyses of tumor tissues reveal upregulation of E-cadherin and downregulation of Snail and phospho-MAPK, confirming the reversal of EMT and inhibition of MAPK signaling with AuNP treatment. The ability of a single self-therapeutic nanoparticle to abrogate signaling cascades of multiple growth factors is distinctive and has potential medical applications as an antitumor/antimetastatic agent.

9.4.2.4 Nanoshell-Based Cancer Therapy

Nanoshells may be combined with targeting proteins and used to ablate target cells. This procedure can result in the destruction of solid tumors or possibly metastases not otherwise observable by the oncologist. In addition, nanoshells can be utilized to reduce angiogenesis present in cancer. Experiments in animals, in vitro and in tissue, demonstrate that specific cells (e.g., cancer cells) can be targeted and destroyed by an amount of infrared light that is otherwise not harmful to

surrounding tissue. This procedure may be performed using an external (outside the body) infrared laser. Prior research has indicated the ability to deliver the appropriate levels of infrared light at depths of up to 15 cm, depending upon the tissue. Photothermal tumor ablation in mice has been achieved by using near-infrared-absorbing nanoparticles. The advantages of nanoshell-based tumor cell ablation include:

- Targeting to specific cells and tissues to avoid damage to surrounding tissue.
- Superior side effect profile than targeted chemotherapeutic agents or PDT.
- Repeatability because of:
  - No "tissue memory" as in radiation therapy
  - Biocompatibility
  - Ability to treat metastases and inoperable tumors
- Nanoshells enable a seamless integration of cancer detection and therapy.

9.4.2.5 Nanobody-Based Cancer Therapy

A nanobody with subnanomolar affinity for the human tumor-associated carcino-embryonic antigen (CEA) has been identified (Cortez-Retamozo et al. 2004). This nanobody was conjugated to *Enterobacter cloacae* beta-lactamase, and its site-selective anticancer prodrug activation capacity was evaluated. The conjugate was readily purified in high yields without aggregation or loss of functionality of the constituents. In vitro experiments showed that the nanobody–enzyme conjugate effectively activated the release of phenylenediamine mustard from the cephalosporin nitrogen mustard prodrug 7-(4-carboxybutanamido) cephalosporin mustard at the surface of CEA-expressing LS174T cancer cells. In vivo studies demonstrated that the conjugate had an excellent biodistribution profile and induced regressions and cures of established tumor xenografts. The easy generation and manufacturing yield of nanobody-based conjugates together with their potent antitumor activity make nanobodies promising vehicles for new generation cancer therapeutics.

9.4.3 Nanoparticles Combined with Physical Agents for Tumor Ablation

Several physical agents have been used for ablation of tumors. Nanoparticles can be combined with these techniques, and some examples are shown here.

9.4.3.1 Boron Neutron Capture Therapy Using Nanoparticles

Boron carbide nanoparticles have potential application as a system for T-cell-guided BNCT. In vitro thermal neutron irradiation of melanoma cells incubated with

sub-100-nm nanoparticles induces complete cell death, whereas nanoparticles alone induce no toxicity. A cancer therapeutic plus diagnostic has been developed that is a variation of BNCT using radio-activate boron nitride (BN) nanotubes. BNs are covalently bound to tumor-cloned antibodies or immunoglobulins (IgGs) to deliver intense, short-lived, therapeutic doses of radiation specifically to active tumor sites. The therapy involves activation of the BN nanotubes with a neutron beam (as in BNCT) once the IgG carrier molecules reach their target tissue. In contrast to conventional BNCT, instant BN nanotubes can deliver significant numbers of boron atoms (100–1,000 s) specifically to the tumor site while avoiding exposures to surrounding tissue. BNCT is a technique that relies on (nonradioactive) 10B delivery specifically to a tumor site and then activating it using an accurate beam of epithermal neutrons (low-energy neutrons with velocities adjusted to penetrate tissue to the specific tumor depth where the 10B has lodged). BN nanotube structure is similar to the “rolled-up-graphite” structure of a CNT, six member rings but with boron atoms bound to three surrounding nitrogen atoms and the nitrogen atoms bound to surrounding boron atoms (no conjugation). Thus, each BN nanotube is composed of a substantial number of boron atoms, e.g., 50 %, meaning hundreds to thousands for each nanotube. Boron has a relatively large radioactive cross section and can be easily made radioactive in a neutron flux.

9.4.3.2 Gold Nanoparticles Combined with Radiation Therapy

High atomic number metals, such as gold, preferentially absorb much more X-ray energy than soft tissues and thus augment the effect of ionizing radiation when delivered to cells. Proteins that regulate poly-SUMO (small ubiquitin-like modifier) chain conjugates play important roles in cellular response to DNA damage, such as those caused by cancer radiation therapy. A study has demonstrated that conjugation of a weak SUMO-2/3 ligand to gold nanoparticles (AuNPs) facilitates selective multivalent interactions with poly-SUMO-2/3 chains leading to efficient inhibition of poly-SUMO-chain-mediated protein–protein interactions (Li et al. 2012). The ligand–gold particle conjugate significantly sensitized cancer cells to radiation but was not toxic to normal cells. This study demonstrates a viable approach for selective targeting of poly-Ubl chains through multivalent interactions created by nanoparticles that can be chosen based on their properties, such as abilities to augment radiation effects.

A method for the targeting of gold nanoparticles to a tumor in a mouse model is based on the use of the pH low insertion peptide (pHLIP), which delivers various imaging agents to acidic tumors (Yao et al. 2013). A comparison of tumor targeting by nonfunctionalized nanogold particles with nanogold–pHLIP conjugates, where nanogold is covalently attached to the N terminus of pHLIP, shows that both intratumoral and IV administration demonstrated a significant enhancement of tumor uptake of gold nanoparticles conjugated with pHLIP. Statistically significant reduction of gold accumulation was observed in acidic tumors and kidney when pH-insensitive K-pHLIP was used as a vehicle, suggesting an important role of pH in the

pHLIP-mediated targeting of gold nanoparticles. pHLIP technology can substantially improve delivery of gold nanoparticles to tumors by providing specificity of targeting, enhancing local concentration in tumors, and distributing nanoparticles throughout the tumor mass where they remain for an extended period, which can facilitate imaging as well as thermolysis or radiation of tumors. Anticancer drugs may be attached to gold nanoparticle conjugates for delivery.

9.4.3.3 Laser-Induced Cancer Destruction Using Nanoparticles

Biological systems are known to be highly transparent to 700- to 1,100-nm NIR light. It is shown here that the strong optical absorbance of SWCNTs in this special spectral window, an intrinsic property of CNTs, can be used for optical stimulation of nanotubes inside living cells to enable multifunctional nanotube biological transporters. Oligonucleotides transported inside living cells by nanotubes can translocate into cell nucleus upon endosomal rupture triggered by NIR laser pulses. Continuous NIR radiation can cause cell death because of excessive local heating of CNTs in vitro. Selective cancer cell destruction can be achieved by functionalization of CNTs with a folate moiety, selective internalization of CNTs inside cells labeled with folate receptor tumor markers, and NIR-triggered cell death, without harming receptor-free normal cells. Thus, the transporting capabilities of CNTs combined with suitable functionalization chemistry and their intrinsic optical properties can lead to new classes of novel nanomaterials for drug delivery and cancer therapy. One example for application is lymphoma as lymphoma cells have well-defined surface receptors that recognize unique antibodies. When attached to a CNT, the antibody would play the role of a Trojan horse. This approach is being tested in laboratory mice with lymphoma. The researchers want to determine if shining NIR on the animal's skin will destroy lymphatic tumors, while leaving normal cells intact. CNTs also can be delivered to diseased cells by direct injection. The idea is to use the nanotube to deliver therapeutic molecules of DNA, RNA, or protein directly into the cell nucleus to fight various infections and diseases.

Plasmon-resonant gold nanorods, which have large absorption cross sections at near-infrared frequencies, are excellent candidates as multifunctional agents for image-guided therapies based on localized hyperthermia. The controlled modification of the surface chemistry of the nanorods is of critical importance, as issues of cell-specific targeting and nonspecific uptake must be addressed prior to clinical evaluation. Nanorods coated with cetyltrimethylammonium bromide (a cationic surfactant used in nanorod synthesis) are internalized within hours into cancer cells by a nonspecific uptake pathway, whereas the careful removal of cetyltrimethylammonium bromide from nanorods functionalized with folate results in their accumulation on the cell surface over the same time interval. Thus the nanorods render the tumor cells highly susceptible to photothermal damage when irradiated at the nanorods' longitudinal plasmon resonance, generating extensive blebbing of the cell membrane at laser fluences as low as 30 J/cm^2 (Huff et al. 2007).

A light-controlled delivery system that can be tailored to release nonbiological molecules into living cells can be remotely controlled and can release quantifiable amounts on demand. The technique utilizes gold nanoparticles, in the form of nanoshells, to transport the target molecule into the cell, where it can subsequently be released. dsDNA-nanoshells can be loaded with molecules, which are associated with the DNA; these molecules can be released inside the cell when triggered by light (Huschka et al. 2010). The research describes how the nanoshell complexes were loaded with 4′,6-diamino-2-phenylindole (DAPI), a fluorescent blue dye that is able to reversibly bind to DNA. The nanoshells were then introduced to cancer cells, and once uptake of the nanoparticles by the cells was confirmed, the cells were illuminated using a continuous wave laser at a specified wavelength. The wavelength of the laser excitation is tailored to the specific DNA, the plasmon resonance wavelength dehybridizes the DNA, causing the release of the DAPI molecule. The DAPI molecule is released from the nanoshell and diffuses through the cytoplasm into the cell nucleus. The diffusion of the DAPI molecule into the cell nucleus was confirmed by the staining of the nuclear DNA. The ability of DAPI to reversibly stain DNA fluorescent blue allowed the intracellular release process to be easily visualized. The research concluded that the light-triggered release of DAPI using a laser did not have an adverse effect on the cells, due to the low power of the laser and the minimal irradiation times required to stimulate the release of the molecule. The researchers also discerned that the uptake of the nanoshells had no adverse effects on the living cells.

Poly(lactic-co-glycolic acid) nanoparticles can encapsulate the photosensitizer meso-tetraphenylporpholactol and are stable and nonphototoxic upon systemic administration. Upon cellular internalization, the photosensitizer is released from the nanoparticle and becomes highly phototoxic. Irradiation with visible light results in cell-specific killing of several cancer cell lines. In vivo experiments have shown complete eradication of cancers in mouse models. The concept of photosensitizers with selective phototoxicity should have widespread applications in cancer therapy.

A nanocarrier consisting of polymeric micelles of diacylphospholipid-poly(ethylene glycol) (PE-PEG) coloaded with the photosensitizer drug 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH), and magnetic Fe_3O_4 nanoparticles has been used for guided drug delivery together with light-activated PDT for cancer (Cinteza et al. 2006). The nanocarrier shows excellent stability and activity over several weeks. The loading efficiency of HPPH is practically unaffected upon coloading with the magnetic nanoparticles, and its phototoxicity is retained. The magnetic response of the nanocarriers was demonstrated by their magnetically directed delivery to tumor cells in vitro. The magnetophoretic control on the cellular uptake provides enhanced imaging and phototoxicity. These multifunctional nanocarriers demonstrate the exciting prospect offered by nanochemistry for targeting PDT.

In a novel nanoformulation of PDT for cancer, the photosensitizer molecules are covalently incorporated into organically modified silica (ORMOSIL) nanoparticles (Ohulchanskyy et al. 2007). The incorporated photosensitizer molecules retain their

spectroscopic and functional properties and can robustly generate cytotoxic singlet oxygen molecules upon photoirradiation. The synthesized nanoparticles are of ultralow size (~20 nm) and are highly monodispersed and stable in aqueous suspension. The advantage offered by this covalently linked nanofabrication is that the drug is not released during systemic circulation, which is often a problem with physical encapsulation. These nanoparticles are also avidly taken up by tumor cells and demonstrate phototoxic action, thereby improving the diagnosis as well as PDT of cancer.

9.4.3.4 Nanoparticle-Mediated Thermal Ablation of Cancer

Thermal ablation of cancer is a recognized technique and is involved in laser ablation of cancer as well. Hyperthermia is the gentle heating of tissue below ablation temperatures, typically <5 °C above normal body temperature. Clinical studies have demonstrated hyperthermia can more than double the efficacy of radiation therapy in select tumors, without an increase in toxicity, and can enhance the efficacy of a number of chemotherapeutic agents for many types of solid tumors. Thermal ablation of cancer has been refined by use of nanoparticles. Some examples are given in this section, and further examples are given in following sections where combination of imaging with thermolysis is described.

An experimental procedure for the treatment of breast cancer is called magnetic thermal ablation. Magnetic nanoparticles are promising tools for the minimal invasive elimination of small tumors in the breast using magnetically induced heating. The approach complies with the increasing demand for breast-conserving therapies and has the advantage of offering a selective and refined tuning of the degree of energy deposition allowing an adequate temperature control at the target.

Anti-HER2 antibody can induce antitumor responses and can be used in delivering drugs to HER2-overexpressing cancer. Anti-HER2 immunoliposomes containing magnetite nanoparticles, which act as tumor-targeting vehicles, have been used to combine anti-HER2 antibody therapy with hyperthermia in experimental studies. SWCNTs emit heat when they absorb energy from NIR light. Tissue is relatively transparent to NIR, which suggests that targeting SWCNTs to tumor cells, followed by noninvasive exposure to NIR light, will ablate tumors within the range of NIR. One study has demonstrated the specific binding of MAb-coupled SWCNTs to tumor cells in vitro, followed by their highly specific ablation with NIR light (Chakravarty et al. 2008). Only the specifically targeted cells were killed after exposure to NIR light.

Targeted nanotherapeutics (TNT) system is an innovation of thermal ablation of cancer that bonds iron nanoparticles and MAbs into bioprobes. The magnetic field energy is converted to lethal heat by the particles causing a rapid temperature increase to more than 170 °C. at the surface of the cancer cells, killing them and their blood supply with negligible damage to surrounding healthy tissues. To evaluate the potential of TNT for in vivo tumor targeting, efficacy and predictive radionuclide-based heat dosimetry were studied using ^{111}In-ChL6 bioprobes (ChL6

is chimeric L6) in a human breast cancer xenograft model (Denardo et al. 2007). Mice in the study received a series of alternating magnetic field (AMF) bursts in a single 20-min treatment. Dosing was calculated using an equation that included tumor concentration of bioprobes, heating rate of particles at different amplitudes, and the spacing of AMF bursts. MAb-guided bioprobes (iron oxide nanoparticles) effectively targeted the tumors without causing particle-related toxicity. Tumor total heat dose, calculated using empirically observed ^{111}In-bioprobe tumor concentration and in vitro nanoparticle heat induction by AMF, correlated with tumor growth delay. The biggest problem of thermotherapy of cancer has been how to apply it to the tumor alone, how to predict the amount needed, and how to determine its effectiveness. By combining nanotechnology, focused AMF therapy and quantitative molecular imaging techniques, a safe technique has been developed that could be considered for clinical use as a treatment for breast and other cancers.

Metal nanoshells belong to a class of nanoparticles with tunable optical resonances that have been used for thermal ablative therapy for cancer. Nanoshells can be tuned to strongly absorb light in the NIR, where optical transmission through tissue is optimal. Nanoshells placed at depth in tissues can be used to deliver a therapeutic dose of heat by using moderately low exposures of extracorporeally applied NIR. In vivo studies under MRI guidance have revealed that exposure to low doses of NIR in solid tumors treated with metal nanoshells reach temperatures capable of inducing irreversible tumor destruction within minutes. Gold nanoshells are ~120 nm in diameter, and a cancer cell is 170 times bigger. Therefore, nanoshells can penetrate the tumor capillaries and lodge in the tumor. Application of NIR light, which passes through the skin harmlessly, heats the nanoshells and kills the tumor cells. Since no drug is used, the cancer cells are unlikely to develop drug resistance.

The ability to control both wavelength-dependent scattering and absorption of nanoshells offers the opportunity to design nanoshells which provide both diagnostic and therapeutic capabilities in a single nanoparticle. A nanoshell-based all-optical platform technology can integrate cancer imaging and therapy applications. Immunotargeted nanoshells, engineered to both scatter light in the near-infrared range enabling optical molecular cancer imaging and to absorb light, enable selective destruction of targeted carcinoma cells through photothermal therapy. In a proof of principle experiment, dual-imaging/therapy immunotargeted nanoshells were used to detect and destroy breast carcinoma cells that overexpress HER2, a clinically relevant cancer biomarker. This approach has some significant advantages over alternatives that are under development. For example, optical imaging is much faster and less expensive than other medical imaging techniques. Gold nanoparticles are also more biocompatible than other types of optically active nanoparticles, such as QDs.

Nanospectra Biosciences Inc. is already developing nanoshells for the targeted destruction of various cancers using nanoshells (AuroShell™). AuroLase™ cancer therapy combines the unique physical and optical properties of AuroShell™ microparticles with a near-infrared laser source to thermally destroy cancer cells without significant damage to surrounding tissue. AuroShell™ microparticles are

injected intravenously and specifically collect in the tumor through the associated leaky vasculature (the enhanced permeability and retention effect or EPR). After the particles accumulate in a tumor, the area is illuminated with a near-infrared laser at wavelengths chosen to allow the maximum penetration of light through tissue. Unlike solid metals and other materials, AuroShell™ microparticles are designed to specifically absorb this wavelength, converting the laser light into heat. This results in the rapid destruction of the tumor along its irregular boundaries. The basics of this approach have been tested experimentally.

The blood vessels inside tumors develop poorly, allowing small particles like nanoshells to leak out and accumulate inside tumors. An animal trial involved 25 mice with tumors ranging in size from 3 to 5.5 mm. The mice were divided into three groups. The first group was given no treatment. The second received saline injections, followed by 3 min exposure to near-infrared laser light. The final group received nanoshell injections and laser treatments. In the test, researchers injected nanoshells into the mice, waited 6 h to give the nanoshells time to accumulate in the tumors and then applied a 5 mm laser beam on the skin above each tumor. Surface temperature measurements taken during the laser treatments showed a marked increase that averaged about 46° F (7.7° C) for the nanoshells group. There was no measurable temperature increase at the site of laser treatments in the saline group. Likewise, sections of laser-treated skin located apart from the tumor sites in the nanoshells group also showed no increase in temperature, indicating that the nanoshells had accumulated as expected within the tumors. All signs of tumors disappeared in the nanoshells group within 10 days. These mice remained cancer-free after treatment. Tumors in the other two test groups continued to grow rapidly. All mice in these groups were euthanized when the tumors reached 10 mm in size. The mean survival time of the mice receiving no treatment was 10.1 days; the mean survival time for the group receiving saline injections and laser treatments was 12.5 days. The advantages of nanoshell-based tumor cell ablation include:

- Targeting to specific cells and tissues to avoid damage to surrounding tissue
- Less adverse effects than targeted chemotherapeutic agents or PDT
- Repeatability because of lack of "tissue memory" as in radiation therapy and biocompatibility
- Ability to treat malignancies such as glioblastoma multiforme, metastases, and inoperable tumors

Thermosensitive liposomes have been used as vehicles for the delivery and release of drugs to tumors. To improve the targeting efficacy for breast cancer treatment, a HER2-specific affibody molecule was conjugated to the surface of thermosensitive small unilamellar liposomes of measuring 80–100 nm referred to as "Affisomes," to study effects of this modification on physical characteristics and stability of the resulting preparation (Puri et al. 2008). Affisomes released calcein, a water-soluble fluorescent probe, in a temperature-dependent manner, with optimal leakage (90–100 %) at 41° C. Affisomes, when stored at room temperature, retained >90 % entrapped calcein up to 7 days. Affisomes are promising candidates for targeted thermotherapy of breast cancer.

Actium Cancer Treatment System (Actium Biosystems) that uses externally generated magnetic energy to heat magnetic nanoparticles embedded in or adjacent to tumors that in combination with radiation, chemotherapy, or immunotherapy produces a therapeutic benefit. It is being applied to the treatment of bladder cancer.

9.4.3.5 Ultrasound Radiation of Tumors Combined with Nanoparticles

Nanoparticles have been introduced in tumors followed by ultrasound-induced cavitation for safe and efficient drug and gene delivery. In a study on athymic nude mice bearing human colon KM20 tumors, polystyrene nanoparticles (100 and 280 nm in diameter) were injected intravenously in combination with ultrasound to enhance delivery of chemotherapeutic agent 5-FU (Larina et al. 2005). This combination significantly decreased tumor volume and resulted in complete tumor regression at optimal irradiation conditions.

9.4.4 Impact of Nanotechnology-Based Imaging in Management of Cancer

The role of nanotechnology in diagnostic imaging of cancer, particularly MRI, has already been described earlier in this chapter. Nanotechnology-based cancer imaging will lead to sensitive and accurate detection of early stage cancer. Nanoparticle-enabled imaging can help accurate delivery of cancer therapy.

9.4.4.1 Cornell Dots for Cancer Imaging

Cornell dots (C dots) are ultrasmall, cancer-targeted, multimodal silica nanoparticle <7 nm in diameter, which has been surface-functionalized with cyclic arginine–glycine–aspartic acid peptide ligands and radioiodine. C dots exhibit high-affinity binding, favorable tumor-to-blood residence time ratios, and enhanced tumor-selective accumulation in αvβ3 integrin-expressing melanoma xenografts in mice (Benezra et al. 2011). The silica shell, essentially glass, is chemically inert and small enough to pass through the body and out in the urine. Coating the dots by PEGylation further protects them from being recognized by the body as foreign substances, giving them more time to find targeted tumors. The outside of the shell can also be coated with organic molecules that can attach to desired targets on tumor surfaces or within tumors. The cluster of dye molecules in a single dot fluoresces under near-infrared light much more brightly than single dye molecules, and the fluorescence identifies malignant cells, showing a surgeon exactly what needs to be cut out and helping ensure that all malignant cells are found. C dots can reveal the extent of a tumor's blood vessels, cell death, treatment response, and invasive or metastatic spread to lymph nodes and distant organs. The FDA has approved the

first clinical trial in humans of C dots that can light up cancer cells in PET-optical imaging. The technology aims to safely show surgeons extent of tumors in human organs. The trial is being conducted at Memorial Sloan-Kettering Center, New York. Commercial development will be in collaboration with Hybrid Silica Technologies Inc.

9.4.4.2 Nanoparticle-MRI for Tracking Dendritic Cells in Cancer Therapy

Several techniques have been developed that allow an effective cellular internalization of clinical superparamagnetic iron oxide (SPIO) formulations without affecting cell proliferation, differentiation, and function, with "magnetoelectroporation" being the most recent labeling paradigm. Animal studies have shown that the MR distribution pattern is reliable when cells have limited cell division, as validated by conventional histological techniques. Magnetically labeled stem cells are not yet in clinical use due to safety concerns about the in vivo behavior of stem cells. A phase I trial has shown the feasibility and safety of imaging autologous dendritic cells that were labeled with a clinical superparamagnetic iron oxide formulation or ^{111}In-oxine and were co-injected intranodally in melanoma patients under ultrasound guidance. In contrast to scintigraphic imaging, MRI allowed assessment of the accuracy of dendritic cell delivery and of inter- and intranodal cell migration patterns. MRI cell tracking using iron oxides appears clinically safe and well suited to monitor cellular therapy in humans. It is believed that MRI cell tracking will become an important technique that someday may become routine in standard radiological practice once stem cell therapy enters clinical practice.

9.4.4.3 Nanoparticle CT Scan

Use of nanomaterials for one of the most common imaging techniques, computed tomography (CT), has remained unexplored. Current CT contrast agents are based on small iodinated molecules. They are effective in absorbing X-rays, but nonspecific distribution and rapid pharmacokinetics have rather limited their microvascular and targeting performance. While most of the nanoparticles are designed to be used in conjunction with MRI, bismuth sulfide (Bi_2S_3) nanoparticles naturally accumulate in lymph nodes containing metastases and show up as bright white spots in CT images (Rabin et al. 2006). A polymer-coated Bi_2S_3 nanoparticle preparation has been proposed as an injectable CT imaging agent. This preparation demonstrates excellent stability at high concentrations, high X-ray absorption (fivefold better than iodine), very long circulation times (>2 h) in vivo, and an efficacy/safety profile comparable to or better than iodinated imaging agents. The utility of these polymer-coated Bi_2S_3 nanoparticles for enhanced in vivo imaging of the vasculature, the liver, and lymph nodes has been demonstrated in mice. These nanoparticles and their bioconjugates are expected to become an important adjunct to in vivo imaging

of molecular targets and pathological conditions. Tumor-targeting agents are now being added to the surfaces of these polymer-coated Bi_2S_3 nanoparticles.

9.4.4.4 QDs Aid Lymph Node Mapping in Cancer

An improved method for performing sentinel lymph node (SLN) biopsy, which depends on illuminating lymph nodes during cancer surgery, has been developed using QDs that emit NIR light, a part of the spectrum that is transmitted through biological tissue with minimal scattering. SLN mapping is a common procedure used to identify the presence of cancer in a single, “sentinel” lymph node, thus avoiding the removal of a patient’s entire lymph system. SLN mapping relies on a combination of radioactivity and organic dyes, but the technique is inexact during surgery, often leading to removal of much more of the lymph system than necessary, causing unwanted trauma. QD technique is a significant improvement over the dye/radioactivity method currently used to perform SLN mapping. The imaging system and QDs allowed the pathologist to focus on specific parts of the SLN that would be most likely to contain malignant cells, if cancer were present.

Different varieties of PEG-coated QDs have been injected directly into tumors in mouse models of human cancer and their course tracked through the skin using NIR fluorescence microscopy to image and map SLNs (Ballou et al. 2007). In tumors that drained almost immediately to the SLNs, the QDs were confined to the lymphatic system, mapping out the connected string of lymph nodes. This provided easy tagging of the SLNs for pathology and there was little difference in results among the different QD types used. Examination of the SLNs identified by QD localization showed that at least some contained metastatic tumor foci. The animals used in this study were followed for >2 years, with no evidence of toxicity, even though QDs could still be observed within the animals. SLN mapping has already revolutionized cancer surgery. NIR QDs have the potential to improve this important technique even further. Because the QDs in the study are composed of heavy metals, which can be toxic, they have not yet been approved for clinical use until safety has been established.

9.4.4.5 Nanosensor Device as an Aid to Cancer Surgery

Scientists at the University of Nebraska–Lincoln have developed a high-resolution touch sensor, one that uses a self-assembling nanoparticle device and acts much like a human finger. The self-assembly process developed by the research team involves no complex lithography, thus proving to be cost-effective and would be relatively easy to reproduce. This device has the ability to sense texture by touch, which is vital for surgeons who need the “touch sensation” in order to operate with precision and accuracy, such as when it comes to detecting and removing cancer cells from the body. One of the most important applications of this newly created sensor is the potential it holds for cancer surgeons, who are faced with the difficult task of

knowing where to stop cutting when removing cancer cells in the body. In the development of artificial skin, the nanodevice structure can attain resolution of ~20 μm or even less. As this dimension is comparable to single-cell dimension, one can hope to “see” a single cancer cell in a tissue. The next goal is to make a high-resolution thermal imaging device and develop an ultrasound detector with a much better image resolution to enable detection of malignant tumors at early stages.

9.4.4.6 Role of Nanoparticle-Based Imaging in Oncology Clinical Trials

Currently CT scans are used as surrogate endpoints in cancer clinical trials. The size of the tumor gives only limited information about the effectiveness of therapy. New imaging agents could speed the clinical trials process in two ways: (1) better imaging data could help oncologists better select which therapies to use on a particular patient, and (2) increasingly sensitive and specific imaging agents will be able to provide real-time information about whether a therapy is working. Currently, oncologists and their patients must wait months to determine if a given therapy is working. Shorter clinical trials would mean that effective new drugs would reach patients quicker and ineffective drugs would be dropped from clinical trials sooner, allowing drug discoverers to better focus their efforts on more promising therapies.

9.4.5 Nanoparticle-Based Anticancer Drug Delivery to Overcome MDR

Although MDR is known to develop through a variety of molecular mechanisms within the tumor cell, many tend to converge toward the alteration of apoptotic signaling. The enzyme glucosylceramide synthase (GCS), responsible for bioactivation of the proapoptotic mediator ceramide to a nonfunctional moiety glucosylceramide, is overexpressed in many MDR tumor types and has been implicated in cell survival in the presence of chemotherapy.

A study has to investigated the therapeutic strategy of coadministering ceramide with paclitaxel in an attempt to restore apoptotic signaling and overcome MDR in the human ovarian cancer cell line using modified poly(epsilon-caprolactone) (PEO–PCL) nanoparticles to encapsulate and deliver the therapeutic agents for enhanced efficacy (van Vlerken et al. 2007). Results show that indeed the complete population of MDR cancer cells can be eradicated by this approach. Moreover, with nanoparticle drug delivery, the MDR cells can be resensitized to a dose of paclitaxel near the IC50 of non-MDR (drug sensitive) cells, indicating a 100-fold increase in chemosensitization via this approach. Molecular analysis of activity verified the hypothesis that the efficacy of this therapeutic approach is due to a restoration in apoptotic signaling, although the beneficial properties of PEO–PCL nanoparticle delivery enhanced the therapeutic success even further, showing the promising potential for the clinical use of this therapeutic strategy to overcome MDR.

Besides MDR, this novel paclitaxel–ceramide nanoparticle therapy also shows great potential for use in the treatment of non-MDR cancer types, in which therapeutic efficacy of paclitaxel is also enhanced.

Nanotechnology, used in conjunction with existing therapies, such as gene therapy and P-glycoprotein inhibition, has been shown to improve the reversal of drug resistance. The mechanisms involved include specific targeting of drugs, enhanced cellular uptake of drugs, and improved bioavailability of drugs. Important strategies in the reversal of drug resistance include (Palakurthi et al. 2012):

- A multifunctional nanoparticulate system
- Therapeutics to kill resistant cancer cells and cancer stem cells
- Release of encapsulated cytotoxic therapeutics in a stimuli-responsive tumor microenvironment

9.4.6 Nanoparticles for Targeting Tumors

Nanoparticles can deliver chemotherapy drugs directly to tumor cells and then give off a signal after the cells are destroyed. Drugs delivered this way are 100 times more potent than standard therapies. Gold nanoparticles can help X-rays kill cancerous cells more effectively in experiments on mice. Combination of nanoparticles followed by X-ray treatment reduced the size of the tumors, or completely eradicated them, whereas tumors that had received only X-ray therapy continued to grow. The gold nanoparticles had no therapeutic effect on their own. The technique works because gold, which strongly absorbs X-rays, selectively accumulates in tumors. This increases the amount of energy that is deposited in the tumor compared with nearby normal tissue.

Efficient conversion of strongly absorbed light by plasmonic gold nanoparticles to heat energy and their easy bioconjugation suggest their use as selective photothermal agents in molecular cancer cell targeting (El-Sayed et al. 2006). Two oral squamous carcinoma cell lines and one benign epithelial cell line were incubated with anti-epithelial growth factor receptor (EGFR) antibody-conjugated gold nanoparticles and then exposed to continuous visible argon ion laser at 514 nm. Malignant cells required less than half the laser energy to be killed than the benign cells after incubation with anti-EGFR antibody-conjugated Au nanoparticles. No photothermal destruction was observed for all types of cells in the absence of nanoparticles at four times energy required to kill the malignant cells with anti-EGFR/Au conjugates bonded. Au nanoparticles thus offer a novel class of selective photothermal agents using a continuous wave (CW) laser at low powers. The ability of gold nanoparticles to detect cancer was demonstrated previously. Now it will be possible to design an “all-in-one” active agent that can be used to noninvasively find the cancer and then destroy it. This selective technique has a potential in molecularly targeted photothermal therapy in vivo.

9.4.7 Nanocarriers with TGF-β Inhibitors for Targeting Cancer

TGF-β inhibitors can prevent the growth and metastasis of certain cancers. However, there may be adverse effects caused by TGF-β signaling inhibition, including the induction of cancers by the repression of TGF-β-mediated growth inhibition. Application of a short-acting, small-molecule TGF-β type I receptor (TR-I) inhibitor at a low dose has been shown to be effective in treating several experimental intractable solid tumors, including pancreatic adenocarcinoma and diffuse-type gastric cancer, characterized by hypovascularity and thick fibrosis in tumor microenvironments. Low-dose TR-I inhibitor alters neither TGF-β signaling in cancer cells nor the amount of fibrotic components (Kano et al. 2007). However, it decreases pericyte coverage of the endothelium without reducing endothelial area specifically in tumor neovasculature and promotes accumulation of macromolecules, including anticancer nanocarriers, in the tumors. Compared with the absence of TR-I inhibitor, anticancer nanocarriers exhibit potent growth-inhibitory effects on these cancers in the presence of TR-I inhibitor. The use of TR-I inhibitor combined with nanocarriers may thus be of significant clinical and practical importance in treating intractable solid cancers.

9.4.8 Nanobombs for Cancer

Nanobombs are nanoscale bombs, which infiltrate into tumors in a minimally invasive manner and then explode on exposure to physical or chemical triggers. Various nanomaterials have been used for the construction of nanobombs including gold and silica nanoparticles as well as carbon nanotubes. Nanobombs are effective anticancer agents as the shock waves that are generated after local explosion inside the tumor kill cancer cells and also disrupt cancer pathways so that the effect spreads beyond the area of explosion.

Temperature change can be used to trigger explosion. Nanogels fabricated by light cross-linking exhibit abrupt volume expansion upon exposure to sudden temperature change, causing cell death (Lee et al. 2009). In another approach, nanoclusters (gold nanobombs) can be activated in cancer cells only by confining near-infrared laser pulse energy within the critical mass of the nanoparticles in the nanocluster (Zharov et al. 2005). Once the nanobombs are exploded and kill cancer cells, macrophages can effectively clear the cell debris and the exploded nanotube along with it.

Blending of supramolecular chemistry and mechanostereochemistry with MSNs has led to a new class of materials that are biological nanoscale bombs with the potential to infiltrate cells and explode upon the pulling of a chemical trigger (Cotí et al. 2009). The triggers are initiated by changes in pH, light, and redox potentials, in addition to enzymatic catalysis. This approach has been tried in

"in vitro" experiments where loaded mechanized silica nanoparticles are endocytosed selectively by cancer cells, and an intracellular trigger causes release of a cytotoxin, effectively leading to apoptosis.

9.4.9 Combination of Diagnostics and Therapeutics for Cancer

9.4.9.1 Aptamer-Conjugated Magnetic Nanoparticles

Magnetic nanoparticles have shown promise for targeted drug delivery, hyperthermia, and MRI imaging in cancer. Aptamer-conjugated magnetic nanoparticles controlled by an externally applied 3D rotational magnetic field have been developed as a nanosurgical approach for the removal of cancerous cells selectively from the interior of an organ or tissue without any collateral damage (Nair et al. 2010). This system could be upgraded for the selective removal of complex cancers from diverse tissues by incorporating various target-specific ligands on magnetic nanoparticles.

9.4.9.2 Biomimetic Nanoparticles Targeted to Tumors

Nanoparticle-based diagnostics and therapeutics hold great promise because multiple functions can be built into the particles. One such function is an ability to home to specific sites in the body. Biomimetic particles that not only home to tumors but also amplify their own homing have been described (Simberg et al. 2007). The system is based on a peptide that recognizes clotted plasma proteins and selectively homes to tumors, where it binds to vessel walls and tumor stroma. Iron oxide nanoparticles and liposomes coated with this tumor-homing peptide accumulate in tumor vessels, where they induce additional local clotting, thereby producing new binding sites for more particles. The system mimics platelets, which also circulate freely but accumulate at a diseased site and amplify their own accumulation at that site. The self-amplifying homing is a novel function for nanoparticles. The clotting-based amplification greatly enhances tumor imaging, and the addition of a drug carrier function to the particles is envisioned.

9.4.9.3 Dendrimer Nanoparticles for Targeting and Imaging Tumors

Dendrimer nanoparticles have been used to entrap metal nanoparticles, a combination that could serve as a potent imaging and thermal therapy agent for tumors if it were not for associated toxicity issues. To eliminate the toxicity associated with dendrimer–metal nanoparticle combinations, methods have been developed for modifying the surface of dendrimers laden with gold nanoparticles. This chemical treatment greatly reduces the toxicity of the hybrid nanoparticle without changing

its size. Construction of novel dendrimers with biocompatible components and the surface modification of commercially available dendrimers by PEGylation, acetylation, glycosylation, and amino acid functionalization have been proposed to solve the safety problem of dendrimer-based nanotherapeutics (Cheng et al. 2011). There are several opportunities and challenges for the development of dendrimer-based nanoplatforms for targeted cancer diagnosis and therapy.

9.4.9.4 Gold Nanoparticle Plus Bombesin for Imaging and Therapy of Cancer

Bombesin (BBN) peptides have demonstrated high affinity toward gastrin-releasing peptide (GRP) receptors in vivo that are overexpressed in prostate, breast, and small-cell lung carcinoma. In vivo studies using gold nanoparticles (AuNPs)–BBN and its radiolabeled surrogate ^{198}AuNP–BBN constructs are GRP-receptor-specific showing accumulation with high selectivity in GRP-receptor-rich prostate tumors implanted in severe combined immunodeficient mice (Chanda et al. 2010). The intraperitoneal mode of delivery was found to be efficient as AuNP–BBN conjugates showed reduced RES organ uptake with concomitant increase in uptake at tumor targets. The selective uptake of this new generation of GRP-receptor-specific AuNP–BBN peptide analogs has clinical potential in molecular imaging using CT techniques as the contrast numbers in prostate tumor sites are several fold higher as compared to the pretreatment group. They also provide synergistic advantages by combining molecular imaging with therapy of cancer.

9.4.9.5 Gold Nanorods for Diagnosis Plus Photothermal Therapy of Cancer

Photothermal therapy is based on the enhancement of electromagnetic radiation by noble metal nanoparticles due to strong electric fields at the surface. The nanoparticles also absorb laser light more easily, so that the coated malignant cells only require half the laser energy to be killed compared to the benign cells. This makes it relatively easy to ensure that only the malignant cells are being destroyed. These unique properties provide the potential of designing novel optically active reagents for simultaneous molecular imaging and photothermal cancer therapy. Gold nanorods with suitable aspect ratios (length divided by width) can absorb and scatter strongly in the NIR region (650–900 nm). Changing the spheres into rods lowers the frequency to which the nanoparticles respond from the visible light spectrum used by the nanospheres to the NIR spectrum. Since these lasers can penetrate deeper under the skin than lasers in the visible spectrum, they can reach tumors that are inaccessible to visible lasers.

In vitro studies have demonstrated that gold nanorods are novel contrast agents for both molecular imaging and photothermal cancer therapy (Huang et al. 2006). Nanorods are synthesized and conjugated to anti-epidermal growth factor receptor

(anti-EGFR) monoclonal antibodies (MAbs) and incubated in cancer cell cultures. The anti-EGFR antibody-conjugated nanorods bind specifically to the surface of the malignant-type cells with a much higher affinity due to the overexpressed EGFR on the cytoplasmic membrane of the malignant cells. As a result of the strongly scattered red light from gold nanorods in dark field, observed using a laboratory microscope, the malignant cells are clearly visualized and diagnosed from the non-malignant cells. It is found that, after exposure to continuous red laser at 800 nm, malignant cells require about half the laser energy to be photothermally destroyed than the nonmalignant cells. Thus, both efficient cancer cell diagnostics and selective photothermal therapy are realized at the same time.

9.4.9.6 Magnetic Nanoparticles for Imaging as well as Therapy of Cancer

Several multifunctional nanoparticles are being developed for simultaneous imaging and therapeutic applications in cancer. Tumor-targeting dendrimers can contain an imaging as well as a delivery agent for drugs, genetic materials. A dendrimer linked to a fluorescent imaging agent and paclitaxel can identify tumor cells and kill them simultaneously.

In ovarian cancer, metastasis occurs when cells slough off the primary tumor and float free in the abdominal cavity. If one could use the magnetic nanoparticles to trap drifting cancer cells and pull them out of the abdominal fluid, it may be possible to predict and perhaps prevent metastasis. With this aim, magnetic cobalt spinel ferrite nanoparticles, which have cobalt-spiked magnetite at their core, were coated with biocompatible polygalacturonic acid and functionalized with ligands specific for targeting expressed EphA2 receptors on ovarian cancer cells (Scarberry et al. 2008). By using such magnetic nanoparticle–peptide conjugates, targeting and extraction of malignant cells were achieved with a magnetic field. The particles, which are just 10 nm or less in diameter, are not magnetic most of the time, but when a magnet is present, they become strongly attracted to it. Targeting ovarian cancer cells with receptor-specific peptide-modified magnetic nanoparticles resulted in cell capture from a flow stream in vitro and from the peritoneal cavity of mice in vivo. Successful removal of metastatic cancer cells from the abdominal cavity and from circulation using magnetic nanoparticle conjugates indicates the feasibility of a dialysis-like treatment and may improve long-term survival rates of ovarian cancer patients. This approach can be applied for treating other cancers, such as leukemia, once the receptors on malignant cells are identified and the efficacy of targeting ligands is established. This technique will provide a way to test for and even treat metastatic ovarian cancer. Although the nanoparticles were tested inside the bodies of mice, it is possible to construct an external device that would remove a patient's abdominal fluid, magnetically filter out the cancer cells, and then return the fluid to the body. After surgery for removal of the primary tumor, a patient would undergo such a treatment to remove any residual cancer cells. The researchers are currently developing such a filter and testing it on abdominal fluid from human ovarian cancer patients.

9.4.9.7 Micelles for Targeted Drug Delivery and PET Imaging in Cancer

H40-DOX-cRGD, a multifunctional unimolecular micelle made of a hyperbranched amphiphilic block copolymer with attached doxorubicin (DOX), was tested for targeted anticancer drug delivery and PET imaging in tumor-bearing mice (Xiao et al. 2006). A uniform size distribution and pH-sensitive drug release behavior was observed. There was a much higher cellular uptake in U87MG human glioblastoma cells due to integrin αvβ3-mediated endocytosis than nontargeted unimolecular micelles (i.e., H40-DOX), thereby leading to a significantly higher cytotoxicity. Thus unimolecular micelles formed by hyperbranched amphiphilic block copolymers integrate passive and active tumor-targeting abilities with pH-controlled drug release. Simultaneous PET imaging for diagnosis provides the basis for personalized cancer therapy.

9.4.9.8 Nanobialys for Combining MRI with Delivery of Anticancer Agents

Although gadolinium has been the dominant paramagnetic metal for MRI contrast, the recent association of this lanthanide with nephrogenic systemic fibrosis, an untreatable disease, has spawned renewed interest in alternative metals for molecular MRI. Manganese was one of the first examples of a paramagnetic contrast material studied in cardiac and hepatic MRI because of efficient site-specific MR T1-weighted molecular imaging. Similar to Ca^{2+} and unlike the lanthanides, manganese is a natural cellular constituent and often a cofactor for enzymes and receptors. Mangafodipir trisodium, a manganese blood pool agent, has been approved as a hepatocyte-specific contrast agent with transient side effects due to de-chelation of manganese from the linear chelate. A self-assembled manganese(III)-labeled nanobialy MRI nanoparticle has been developed for combined diagnosis and delivery of a chemotherapeutic agent (Pan et al. 2008). The "bialy" shape affords increased stability. Nanobialy nanoparticles have been characterized for targeted detection of fibrin, a major biochemical feature of thrombus. A complementary ability of nanobialys to incorporate anticancer compounds with greater than 98 % efficiency and to retain more than 80 % of these drugs after infinite sink dissolution, point to the potential of this platform technology to combine a therapeutic agent with a diagnostic agent.

9.4.9.9 Nanoparticles, MRI, and Thermal Ablation of Tumors

Nanostructures with surface-bound ligands can be used for the targeted delivery and ablation of CRC, the third most common malignancy and the second most common cause of cancer-related mortality in the USA. Normal colonic epithelial cells as well as primary CRC and metastatic tumors all express a unique surface-bound guanylyl cyclase C (GC-C), which binds the bacterial heat-stable enterotoxin (ST)—a

peptide. This makes GC-C a potential target for metastatic tumor ablation using ST-bound nanoparticles in combination with thermal ablation with near-infrared or radio-frequency energy absorption (Fortina et al. 2007). Furthermore, the incorporation of iron or iron oxide nanoparticles into such structures would provide advantages for MRI.

Gold nanoshell-based, targeted, multimodal contrast agents in the near-IR are fabricated and utilized as a diagnostic and therapeutic probe for MRI, fluorescence optical imaging, and photothermal cancer therapy of breast carcinoma cells in vitro (Bardhan et al. 2009). This may enable diagnosis as well as treatment of cancer during one hospital visit.

In the future, it may be possible for a patient to be screened for breast cancer using MRI techniques with engineered enhanced ferrites as the MRI contrast agent. Enhanced ferrites are a class of ferrites that are specially engineered to have enhanced magnetic or electrical properties and are created through the use of core-shell morphology. Magnetic nanoparticles are coupled to the radio frequency of the MRI, which converts the radio frequency into heat. If a tumor is detected, the physician could increase the power to the MRI coils and localized heating would destroy the tumor without damage to the surrounding healthy cells. The only hindrance to the development of enhanced ferrites for 100-MHz applications is a lack of understanding of the growth mechanisms and synthesis–property relationships of these nanoparticles. By studying the mechanism for the growth of the enhanced ferrites, it will be possible to create shells that help protect the metallic core from oxidation in biologically capable media.

9.4.9.10 pHLIP Nanotechnology for Detection and Targeted Therapy of Cancer

The pH-selective insertion and folding of a membrane peptide, pHLIP, can be used to target acidic tissue in vivo, including acidic foci in tumors. pHLIP nanotechnology is considered to be a promising approach for mapping areas of elevated acidity in the body. The peptide has three states: soluble in water, bound to the surface of a membrane, and inserted across the membrane. At physiological pH, the equilibrium is toward water, which explains its low affinity for cells in healthy tissue; at acidic pH, the equilibrium shifts toward membrane insertion and tissue accumulation. This peptide acts like a nanosyringe to deliver tags or therapy to cells. Tumors can be detected by labeling pHLIP peptide with Cy5.5 and imaging by use of NIR fluorescence with wavelengths in the range of 700–900 nm. In a mouse breast adenocarcinoma model, fluorescently labeled pHLIP detects solid acidic tumors with high accuracy and accumulates in them even at a very early stage of tumor development (Andreev et al. 2007). The fluorescence signal is stable and is approximately five times higher in tumors than in healthy counterpart tissue. Tumor targeting is based on the fact that most tumors, even very small ones, are acidic as a result of the way they grow, known as the Warburg effect (Nobel Prize 1931). Tumors may be treated by attaching and delivering anticancer agents with pHLIP.

9.4.9.11 QD Conjugates Combine Cancer Imaging, Therapy, and Sensing

The specificity and sensitivity of a QD–aptamer–doxorubicin (QD–Apt–Dox) conjugate as a targeted cancer imaging, therapy, and sensing system has been demonstrated in vitro (Bagalkot et al. 2007). By functionalizing the surface of fluorescent QD with an RNA aptamer, which recognizes the extracellular domain of the PSMA, the system is capable of differential uptake and imaging of prostate cancer cells that express the PSMA. The intercalation of Dox, an anticancer drug with fluorescent properties, in the double-stranded stem of the aptamer results in a targeted conjugate with reversible self-quenching properties based on a Bi-FRET mechanism. A donor–acceptor model FRET between QD and Dox and a donor–quencher model FRET between Dox and aptamer result when Dox is intercalated within the aptamer. This simple multifunctional nanoparticle system can deliver Dox to the targeted prostate cancer cells and sense the delivery of Dox by activating the fluorescence of QD, which concurrently images the cancer cells.

9.4.9.12 Squalene-Based Nanocomposites for Tumor Imaging and Therapy

Nanocomposites, constructed of magnetite nanocrystals into NPs by self-assembling molecules of the squalenoyl gemcitabine (SQgem) bioconjugated, are characterized by an unusually high drug loading, a significant magnetic susceptibility, and a low-burst release. When injected into a subcutaneous mice tumor model, these magnetite/SQgem NPs were magnetically guided and displayed considerably greater anticancer activity than other anticancer treatments including nonmagnetically guided magnetite/SQgem NPs (Arias et al. 2011). The histology and immunohistochemistry investigation of the tumor biopsies clearly evidenced the therapeutic superiority of the magnetically guided nanocomposites, while Prussian blue staining confirmed their accumulation at the tumor periphery. The superior therapeutic activity and enhanced tumor accumulation have been successfully visualized using T2-weighted MRI imaging. This concept was further enlarged by (1) the design of squalene-based NPs containing the T1 $Gd3+$ contrast agent instead of magnetite and (2) the application to other anticancer squalenoyls, such as, cisplatin, doxorubicin, and paclitaxel. This nanotechnology platform is expected to have important applications in imaging-guided cancer therapy.

9.4.9.13 Radiolabeled Carbon Nanotubes for Tumor Imaging and Targeting

SWCNTs with covalently attached multiple copies of tumor-specific MAbs, radiometal-ion chelates, and fluorescent probes can target lymphomas and deliver both imaging and therapeutic molecules to these tumors (McDevitt et al. 2007). Each nanotube, which contained ~6 antibody molecules and 114 radioactive atoms,

proved to be stable in human plasma for at least 96 h and was able to bind to targeted tumor cells. Most importantly, the chemical linkages binding the radioactive element indium-111 was completely stable in human plasma for the entire 4-day experiment. Tests using a mouse model of human lymphoma showed that the nanotube construct successfully targeted tumors while avoiding healthy cells. The ability to specifically target tumor with prototype-radiolabeled or fluorescent-labeled, antibody-appended SWCNT constructs is encouraging and suggests further investigation of these as diagnostic combined with drug delivery for cancer.

9.4.9.14 Ultrasonic Tumor Imaging and Targeted Chemotherapy by Nanobubbles

Drug delivery in polymeric micelles combined with tumor irradiation by ultrasound results in effective drug targeting, but this technique requires prior tumor imaging. A new targeted drug delivery method uses ultrasound to image tumors, while also releasing the drug from nanobubbles into the tumor (Rapoport et al. 2007). Mixtures of drug-loaded polymeric micelles and perfluoropentane (PFP) nanobubbles stabilized by the same biodegradable block copolymer were prepared. Size distribution of nanoparticles was measured by dynamic light scattering. Cavitation activity (oscillation, growth, and collapse of microbubbles) under ultrasound was assessed based on the changes in micelle/nanobubble volume ratios. The effect of the nanobubbles on the ultrasound-mediated cellular uptake of doxorubicin (Dox) in MDA MB231 breast tumors in vitro and in vivo (in mice bearing xenograft tumors) was determined by flow cytometry. Phase state and nanoparticle sizes were sensitive to the copolymer/PFC volume ratio. At physiologic temperatures, nanodroplets converted into nanobubbles. Doxorubicin was localized in the nanobubble walls formed by the block copolymer. Upon intravenous injection into mice, Dox-loaded micelles and nanobubbles extravasated selectively into the tumor interstitium, where the nanobubbles coalesced to produce microbubbles. When exposed to ultrasound, the bubbles generated echoes, which made it possible to image the tumor. The sound energy from the ultrasound popped the bubbles, releasing Dox, which enhanced intracellular uptake by tumor cells in vitro to a statistically significant extent relative to that observed with unsonicated nanobubbles and unsonicated micelles and resulted in tumor regression in the mouse model. In conclusion, multifunctional nanoparticles that are tumor-targeted drug carriers, long-lasting ultrasound contrast agents, and enhancers of ultrasound-mediated drug delivery have been developed and deserve further exploration as cancer therapeutics.

9.4.10 Nanorobotics for Management of Cancer

It is within the realm of possibility to use molecular tools to design a miniature device, e.g., a nanobot that can be introduced in the body, locate, and identify cancer cells and

finally destroy them. The device would have a biosensor to identify cancer cells and a supply of anticancer substance that could be released on encountering cancer cells. A small computer could be incorporated to program and integrate the combination of diagnosis and therapy and provide the possibility to monitor the in vivo activities by an external device. Since there is no universal anticancer agent, the computer program could match the type of cancer to the most appropriate agent. Such a device could be implanted as a prophylactic measure in persons who do not any obvious manifestations of cancer. It would circulate freely and could detect and treat cancer at the earliest stage. Such a device could be reprogrammed through remote control and enable change of strategy if the lesion encountered is other than cancer.

9.4.10.1 Bacterial Nanorobots for Targeting Cancer

Flagellated nanomotors combined with the nanometer-sized magnetosomes of a single magnetotactic bacterium (MTB) can be used as an effective integrated propulsion and steering system for devices such as nanorobots designed for targeting locations only accessible through the smallest capillaries in humans while being visible for tracking and monitoring purposes using modern medical imaging modalities such as MRI (Martel et al. 2009). Through directional and magnetic field intensities, the displacement speeds, directions, and behaviors of swarms of these bacterial actuators can be controlled from an external computer. Such a device can be used for diagnosis as well as therapy of cancer.

9.4.10.2 DNA Robots for Targeting Cancer

DNA nanotechnology is widely investigated for its potential to deliver drugs and molecular signals to cells in the body because DNA is a biocompatible and biodegradable material. However, opinions differ as about the best nanorobot design, i.e., the ideal structure to load, transport, and deliver molecules. Various designs include a spiderlike robot that moves along a chemical track, a nanofactory with mobile robotic walkers and molecular forklifts, and DNA tweezers that open and close to grasp and release molecules.

An autonomous DNA nanorobot has been described that is capable of transporting molecular payloads to cells, sensing cell surface inputs for conditional, triggered activation, and reconfiguring its structure for payload delivery (Douglas et al. 2012). The nanorobot, constructed using a computer-aided design tool called DNA origami, is a hexagonal barrel, 35 nm in diameter, and opens like a clam shell. The device can be loaded with a variety of materials and is controlled by an aptamer-encoded logic gate, enabling it to respond to a wide array of cues that have demonstrated their efficacy in selective regulation of nanorobot function. This barrel-shaped DNA nanorobot seeks out cancer cells and delivers self-destruct instructions. It can successfully deliver antibody fragments to surfaces of cancer cells to kill them and bacterial proteins to activate T cells.

9.4.11 Fullerenes for Protection Against Chemotherapy-Induced Cardiotoxicity

Therapeutic use of doxorubicin as an anticancer drug is limited due to its cardiotoxicity. Generation of free radicals plays an important role in the mechanism of doxorubicin-induced cardiotoxicity. There is significant evidence indicating that mitochondria are the principal targets in this pathological process. Efficacy of fullerenol ($C_{60}OH_{24}$) in preventing single, high-dose doxorubicin-induced cardiotoxicity has been investigated in rats with malignant neoplasm (Injac et al. 2008). Study was performed on adult female Sprague Dawley rats with chemically induced mammary carcinomas. The animals were sacrificed 2 days after the application of doxorubicin and/or fullerenol, and the serum activities of cardiac enzymes were determined. The results obtained showed that the administration of a single dose of 8 mg/kg in all treated groups induces statistically significant cardiotoxicity. There were significant changes in the enzymes lactate dehydrogenase and creatine kinase and increase in level of tissue malondialdehyde (MDA), a product of lipid peroxidation, after intraperitoneal administration of doxorubicin. The results revealed that doxorubicin induced oxidative damage and that the fullerenol antioxidant effect caused significant changes in the levels of biomarker MDA in the heart. Thus fullerenol may have an important role as for cardioprotection in doxorubicin-treated individuals.

9.4.12 Concluding Remarks and Future Prospects of Nanooncology

The rationale for using nanobiotechnology in oncology is that nanoparticles have optical, magnetic, or structural properties that are not available from larger molecules or bulk solids. When linked with tumor-targeting ligands such as MAbs, peptides, or small molecules, nanoparticles can be used to target tumor antigens (biomarkers) as well as tumor vasculatures with high affinity and specificity. In the size range of 5–100-nm diameter, nanoparticles have large surface areas and functional groups for conjugating to multiple diagnostic and therapeutic anticancer agents. Recent advances have led to bioaffinity nanoparticle probes for molecular and cellular imaging, targeted nanoparticle drugs for cancer therapy, and integrated nanodevices for early cancer detection and screening. These developments have provided opportunities for personalized oncology in which biomarkers are used to diagnose and treat cancer based on the molecular profiles of individual patients.

Nanoparticles have shown promise for incorporating multiple functions including diagnosis and therapy of cancer. Most of the work done in this area is still experimental and some challenges need to resolved before clinical applications. These include the following:

- Preventing capture/removal by the reticuloendothelial system
- Difficulties in selective targeting as well as penetration of tumor by systemic administration of anticancer nanostructures, which requires identification of receptors unique to a particular cancer
- Investigation of long-term fate and toxicity concerns of nanoparticles

Efforts are being made to use nanostructures to develop anticancer treatment strategies based on various mitochondrial targets that play vital roles in cancer development and progression. Cancer mitochondria-targeted multifunctional compounds have been identified that could provide an alternative strategy for the development of novel solutions for cancer diagnosis and therapy (Zhang et al. 2011).

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Chapter 10
Cell Therapy of Cancer

10.1 Introduction

Cell therapy is the prevention or treatment of human disease by the administration of cells that have been selected, multiplied, and pharmacologically treated or altered outside the body (ex vivo). The scope of cell therapy can be broadened to include methods, pharmacological as well as non-pharmacological, to modify the function of intrinsic cells of the body in vivo for therapeutic purposes. The aim of cell therapy is to replace, repair, or enhance the function of damaged tissues or organs. The cells used can originate from the patient or from a donor or from another species. Other sources include cell lines and cell from patients' tumors to make cancer vaccines. Cells can be encapsulated in selectively permeable membranes that block entry of immune mediators but allow outward diffusion of active molecules produced by the cells. Genetic engineering of cells is part of ex vivo gene therapy. The cells may be introduced by various routes into the body and selectively implanted at the site of action. More recently, cell therapies have expanded to replace some conventional procedures. Bone marrow (BM) transplants are being replaced by peripheral blood stem cell (PBSC) transplants. Most of the interest in cell therapy centers on stem cells. The reason for the surge of interest in cell therapy is that cells often do a job better than any chemical could. Cell therapy is described in detail in a special report on this topic (Jain 2013).

10.2 Cell Therapy Technologies for Cancer

Various cell therapy technologies used for treatment of cancer are shown in Table 10.1. Some of these are described in the following text.

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_10,

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Table 10.1 Cell therapy technologies used for cancer

Cell therapy technologies
Cell-based delivery of anticancer therapy
Cell replacement as adjunct to chemotherapy
Infusion of peripheral blood stem cells
Bone marrow transplant
Immunomodulatory effects of anticancer agents on T cells
Tyrosine kinase inhibitors, e.g., imatinib
Genetically modified cells secreting anticancer agents
Genetically engineered cells secreting cytokines
Genetically modified encapsulated cells secreting enzymes for activating anticancer prodrugs
Cell-based cancer vaccines
Cytotoxic T cells: hybrid cell vaccination
Tumor-infiltrating lymphocytes
Natural killer (NK) cells
Dendritic cell vaccines
Dendritic cells pulsed with tumor lysate
Dendritic cells pulsed with apoptotic genes
Tumor cell vaccines
Unmodified tumor cell vaccines
Tumor cells fused with dendritic cells
Tumor cells transfected with cytokines
Genetically modified tumor cell vaccines
Stem cell-based anticancer therapies
Nonablative allogeneic hematopoietic stem cell transplantation
Delivery of anticancer agents by genetically engineered MSCs
Hematopoietic progenitor cells with retroviral MDR 1 coexpression vectors

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10.3 Cell-Based Delivery of Anticancer Therapy

Cell-mediated delivery is a therapeutic as well as a diagnostic strategy for cancer. The cytotoxic activity of the cell carriers can be combined with site-specific delivery of anticancer agents, which can be packed into the carriers to reduce cytotoxicity to normal tissues. Nanoparticles have been combined with cell-based delivery systems. A technique has been described to create nanoparticulate cellular patches that remain attached to the membrane of cells for up to 2 days and retain their inherent tumoritropic properties as shown using a tumor model in a 3D extracellular matrix (Cheng et al. 2010). Various cells that have been evaluated as carriers for delivery of anticancer agents include MSCs, RBCs, mononuclear phagocytes, and bacterially derived minicells.

RBCs have been used as carriers for anticancer agent 5-fluorouracil (5-FU) by intravenous injection to treat malignant ascites resulted in an increase of survival time in mice (Wang et al. 2010). Mononuclear phagocytes have been used as "Trojan horses" for gold nanoparticle transport into hypoxic centers of tumors, which were destroyed by irradiation with near-infrared light (Choi et al. 2007). Macrophages can also act as a cellular vehicle for 5-FU encapsulated in oligomannose-coated liposomes

cased in magnetic nanoparticles followed by treatment with an alternating magnetic field, which led to the release of 5-FU from the macrophages. Mononuclear phagocytes can also be used for delivery of therapeutic DNA constructs into tumors.

10.4 Role of Cells in Immunotherapy of Cancer

The immune system is divided into humoral and cellular, according to the type of pathogen the immune system recognizes and can respond to. In humoral immunity, antibodies, which are soluble molecules produced by B lymphocytes, eliminate extracellular pathogens. In cellular immunity, T lymphocytes are designed to eliminate foreign intracellular pathogens and transform cancer cells. Role of T cells, described in Chap. 7 on immunotherapy of cancer, is more specific and less toxic than chemotherapy and radiation. It is now recognized that many cancer patients have tumor-specific T-cell and antibody responses. Most antitumor responses are directed against non-mutated self-antigens. Cellular immunotherapy consists of giving the patient cells that stimulate antitumor activity in the patient (tumor and dendritic cell vaccines) or that have intrinsic antitumor activity (autologous and allogeneic lymphocytes). The aim is to harness potent immunological weapons to destroy cancer cells.

10.4.1 Treatments for Cancer by Ex Vivo Mobilization of Immune Cells

The body's T cells and natural killer (NK) cells need to be mobilized against the cancer. This mobilization is done by dendritic cells, another specialized cell of the immune system. These cells patrol the body in search of foreign cells such as cancer cells. Once detected, the dendritic cells absorb part of the foreign cells' structure and pass this information on to T cells and NK cells, which are then mobilized to kill the cells.

Although NK cells have long been known to mediate antigen-independent tumor cytotoxicity, the therapeutic potential of NK cell-based immunotherapy has yet to be realized. Manipulating the balance between inhibitory and activating NK receptor signals, sensitization of tumor target cells to NK cell-mediated apoptosis, and recent discoveries in NK cell receptor biology have fueled translational research that has led to clinical trials investigating a number of novel methods to potentiate NK cytotoxicity against human malignancies (Srivastava et al. 2008).

10.4.2 Granulocytes as Anticancer Agents

Scientists at the Wake Forest University School of Medicine (Winston-Salem, NC) have shown in laboratory experiments that granulocytes, immune system cells,

from some persons can be almost 50 times more effective in fighting cancer than in others. Granulocyte transfusion has previously been used to try to prevent infections in cancer patients whose immune systems have been weakened by chemotherapy. Now it has been shown that granulocytes have cancer-fighting properties as well and cells from mice found to be immune to cancer can be used to cure ordinary mice with cancer. The work raises the prospect of using granulocytes from donors to significantly boost a cancer patient's ability to fight their disease and possibly cure them. The strength of a person's immune system to combat cancer can vary according to how stressed they are and the time of year. Those of the strongest participants killed close to 97 % of the cancer cells in 24 h, while those of the weakest killed only 2 %. Initial experiments suggest that it may be possible to transfer granulocytes which have demonstrated strong cancer-fighting powers into cancer sufferers.

The FDA has given permission to inject superstrength granulocytes into patients in a pilot trial. The technique can be quickly introduced into the clinic because the technology used to extract granulocytes is the same as that already used by hospitals to obtain other blood components such as plasma or platelets. The use of live cells carries a theoretical risk of graft-versus-host disease (GVHD), which can be fatal. Measures are being considered for minimizing this risk.

10.4.3 Neutrophil Granulocytes in Antibody-Based Immunotherapy of Cancer

Monoclonal antibodies (MAb) are key therapeutic drugs for the treatment of malignancies (see Chap. 8). They can be designed to specifically target tumor-associated antigens and initiate several effector mechanisms, which potentially leads to elimination of cancer. Through their Fc tail, MAbs interact with Fc receptors (FcR) that are expressed on immune cells. Neutrophils are the most abundant circulating FcR-expressing white blood cells with potent cytotoxic ability that is enhanced in the presence of antitumor MAbs. They furthermore play a role in regulating adaptive immunity, which may lead to the initiation of anticancer immune responses. Considerable scientific data that supports the possibility of exploiting neutrophils for MAb-based immunotherapy of cancer and an understanding of this topic may enable future development of new anticancer therapies (van Egmond 2008).

10.5 Stem Cell-Based Anticancer Therapies

Stem cells have two important characteristics that distinguish them from other types of cells. First, they are unspecialized cells that renew themselves for long periods through cell division. The second is that under certain physiological or experimental conditions, they can be induced to become cells with special functions. In the embryo, these cells are the starting point for the development of the complete human

being. In the adult body, stem cells are one of the mechanisms for the repair and renewal of some cells and tissues.

Embryonic stem cells (ESCs) are continuously growing stem cell lines of embryonic origin derived from the pluripotent cells of the inner cell mass or epiblast of the mammalian embryo. They may give rise to any cell type but not to an independent organism. Embryonic germ cell lines, established from primitive reproductive cells of the embryo, are functionally equivalent to ESCs. The distinguishing features of ESCs are their capacity to be maintained in an undifferentiated state indefinitely in culture and their potential to develop into every cell of the body. Their ability to develop into a wide range of cell types makes ESCs a useful basic research tool and a novel source of cell populations for new therapies. Hematopoietic stem cells (HSCs) are of interest in oncology, and some basics are reviewed prior to describing their applications.

10.5.1 Hematopoietic Stem Cells

HSCs, with the capacity to renew, are the source of committed progenitor cells throughout the lifetime of an individual. They differentiate and proliferate to generate large numbers of mature blood cells of all lineages. However, the phenomenon of self-renewal in the absence of differentiation of the HSCs is not well understood. In order to elucidate the underlying mechanisms of hematopoiesis in general, various attempts have been made to establish appropriate in vitro assays facilitating the identification and characterization of hematopoietic stem and progenitor cell populations. Several cytokines and growth factors have been reported to be essential for maintenance or expansion of primitive hematopoietic cells as well as for expansion of more committed progenitor cells during in vitro culture. A general feature of primitive as well as more committed hematopoietic cells is the expression of the CD34 antigen, which can be detected by fluorescence-activated cell sorter (FACS) analysis using anti-CD34 MAbs. Animal models have been established to identify the repopulating SCs by attempting in vivo hematopoietic reconstitution after transplantation of enriched or ex vivo cultured human HSCs.

When HSCs divide, they have one of three fates: (1) develop into two more stem cells, i.e., self-renewal; (2) differentiate to become one of several mature blood cell types; or (3) strike a balance in which one daughter cell becomes an HSC and the other becomes a mature blood cell type. The in vitro differentiation of hESCs provides an opportunity to better understand human hematopoiesis and could lead to a novel source of cells for transfusion and transplantation therapies. In steady state, HSCs remain largely quiescent and self-renew at a constant low rate, forestalling their exhaustion during adult life. Whereas nuclear regulatory factors promoting proliferative programs of HSCs in vivo and ex vivo have been identified, transcription factors restricting their cycling have remained elusive. The zinc-finger repressor Gfi-1 (growth factor independent 1) restricts proliferation of HSCs and preserves their functional integrity, which is relevant to production of stem cells for

therapeutic use. Without the braking effect of Gfi-1, the cells overproliferate but cannot make effective cells.

Over the past 10 years, several gene families have been suggested to be important in regulating HSC fate, e.g., homebox, wnt, notch 1, and telomerase genes. There is a need for discovery of more details of the pathways that regulate the development of HSC in BM as this will provide a better understanding of how HSCs work in the context of BM and peripheral stem cell transplantation. The aim is to find a way to control stem cell fate by biochemically switching on or off at will, to either make more stem cells in the case of BM failure and for transplantation or force the cells to differentiate, in the case of leukemia, where too many HSCs are produced.

Ets-related gene (ERG), which encodes a member of the Ets family of transcription factors, is required for definitive hematopoiesis, adult HSC function, and the maintenance of normal peripheral blood platelet numbers (Loughran et al. 2008). ERG is also a potent oncogene as chromosomal rearrangements involving ERG are found in acute leukemias, Ewing's sarcoma, and more than half of all prostate cancers.

Contrary to the belief that HSCs clustered together somewhere in BM, a study provided compelling visual evidence that HSCs prefer a solitary life (Suzuki et al. 2006). The green fluorescent protein (GFP) reporter gene was spliced into a gene called Gata2, which is known to regulate the activity of HSCs, and into a promoter that specifically controls the expression level of the Gata2 gene in these stem cells. The two modified genes were injected into laboratory mice. It was possible to isolate the HSCs by following the Gata2-directed expression of GFP in mouse BM and another marker protein called Sca1. Specifically, the researchers found that:

- GFP was seen only in immature hematopoietic progenitors that lacked the surface protein markers seen in more mature types of blood cells.
- Only those cells with GFP activity had the ability to reconstitute the BM of mice whose HSCs were destroyed by high doses of radiation.
- HSCs with GFP were immobile and found in contact with bone-forming cells (osteoblasts) at the edge of BM. This location may be important as the microenvironment is a key to maintaining the pluripotency of HSCs.

The discovery has enabled the study of HSCs undisturbed and in their natural environment. This is important because when HSCs are removed from the BM, they either die or start differentiating into different types of specialized blood cells. There is something about the physical location and cellular environment surrounding HSCs in their BM niche that is at least partly responsible for their ability to maintain a primitive, pluripotent state. Now that they can be visualized in vivo, it may be possible to find out how they do it.

Elucidating the mechanisms underlying HSC specification and expansion in the embryo has been hampered by the lack of analytical cell culture systems that recapitulate in vivo development. An ex vivo model has been described that facilitates a rapid and robust emergence of multipotent long-term repopulating HSCs in the embryonic AGM region (Taoudi et al. 2008). Because this method includes a cell

dissociation step prior to reconstruction of a 3D functional tissue and preserves both stromal and hematopoietic elements, it enables identification of the direct ancestry of the rapidly expanding HSC pool. Extensive generation of definitive HSCs was demonstrated in the AGM that occurs predominantly through the acquisition of stem characteristics by the VE-cadherin+CD45+ population. It was possible to multiply HSCs by 150 times.

Hematopoiesis has long served as a model for study of cellular differentiation and its control by underlying gene regulatory networks. The Scl–Gata2–Fli1 triad is a network module essential for the development of HSCs. The transcription factors Scl, Gata2, and Fli1 act in combination to upregulate transcription of each other via distal enhancer site binding. Experimental findings have been used in a method to circumvent the difficulties of mathematically modeling the combinatorial regulation of this triad module (Narula et al. 2010). The results suggest that the Scl–Gata2–Fli1 module possibly functions as a control switch for HSC development. It has all the properties that one would expect to find in a master-level regulator, although this has not been verified.

Another study has shown that function of Runx1, a key transcription factor, is essential in endothelial cells (ECs) for hematopoietic progenitor and HSC formation from the vasculature (Chen et al. 2009). These findings will enable strategies for efficient production of blood stem cells in the laboratory and will be potentially useful for patients in need of BM transplants or blood transfusions.

10.5.1.1 Role of HSCs in the Immune System

HSCs enter the blood circulation, but the ultimate fate and functional relevance of circulating HSCs has been uncertain. The biological role of HSCs in the human body is far more versatile and dynamic than the hitherto known passive role, which implies that HSCs are activated to replenish blood and immune system cells only when called upon. A study has now shown that HSCs can travel from the BM, through the blood system, and enter visceral organs where they perform reconnaissance missions in search of pathogenic invaders (Massberg et al. 2007). Upon encountering an invader, they immediately synthesize a defense, divide and mature, churning out new immune system cells such as dendritic cells and other leukocytes, right on the spot.

The molecular mechanisms underlying these observations have been identified. HSCs' egress from extramedullary tissues into lymph depends on sphingosine-1-phosphate receptors. Migratory HSPCs proliferate within extramedullary tissues and give rise to tissue-resident myeloid cells, preferentially dendritic cells. HSC differentiation is amplified upon exposure to Toll-like receptor agonists. Thus, HSCs can survey peripheral organs and can foster the local production of tissue-resident innate immune cells under both steady-state conditions and in response to inflammatory signals. Thus, the stem cells are actually a part of the immune system, rather than just giving rise to it.

10.5.1.2 Derivation of HSCs from ESCs

HSCs derived from hESCs could provide a therapeutic alternative to BM transplants, but the efficiency of most derivation protocols is low. Coculture with monolayers of cells derived from mouse AGM and fetal liver, or with stromal cell lines derived from these tissues, can enhance hESC hematopoietic differentiation (Ledran et al. 2008). Under such conditions hESC-derived differentiating cells formed early hematopoietic progenitors, with a peak at day 18–21 of differentiation that corresponded to the highest CD34 expression. These hESC-derived HSCs were capable of primary and secondary hematopoietic engraftment into immunocompromised mice at substantially higher levels than described previously. Transcriptional and functional analysis identified TGF-β1 and TGF-β3 as positive enhancers of hESC hematopoietic differentiation that can further stimulate this process when added to the culture. These findings represent significant progress toward the goal of deriving functional HSCs from hESCs.

10.5.2 Stem Cell Transplantation in Cancer

Approximately 45,000 stem cell transplantations are performed yearly worldwide. Stem cell transplantation is used for the treatment of multiple hematological malignancies and refractory solid tumors. The rationales for stem cell transplantation include:

- Giving high doses of chemotherapy or radiation therapy to overcome tumor resistance
- Providing stem cell rescue from the hematological toxicities of chemotherapy
- Replacing abnormal BM

Sources of stem cells include the patient (autologous), an identical twin (syngeneic), an HLA-matched donor (allogeneic), an HLA mismatch or unrelated donor, and umbilical cord blood.

10.5.2.1 Peripheral Blood Stem Cell Transplantation

BM transplants have been used traditionally for BM rescue following chemotherapy. Now peripheral blood is used as a source of these cells. For example, highly purified PBSCs are autografted following myeloablative therapy in patients with lymphoma. HSCs reside predominantly in the BM but can be mobilized in large numbers in the blood by the administration of filgrastim (recombinant granulocyte colony-stimulating factor, G-CSF, Amgen's Neupogen). Advantages of PBSCs compared with autologous BM harvesting are:

- Avoidance of general anesthesia
- A shorter hospital stay

- Decreased use of antibiotics
- Less transfusion needs
- Several studies suggest that the recovery of neutrophils, red cells, and platelets is faster with the use of PBSCs than with the use of BM

Positive clinical results were obtained in a study of transplants of ex vivo-produced human stem cells in breast cancer patients using the AastromReplicell Cell Production System (Aastrom Biosciences) and SC-I Therapy Kit. The results show that this process of stem cell production reduces or eliminates contaminating tumor cells, which may contribute to the long-term recovery results achieved by the patients in this study. In high-dose chemotherapy for the treatment of hematological malignancies, with or without radiation, allogeneic PBSC used for hematopoietic rescue restores blood counts faster than allogeneic BM, without increasing the risk of GVHD. However, a phase III randomized trial did not detect significant 2-year survival differences between PBSC and BM transplantation from unrelated donors (Anasetti et al. 2012). Exploratory analyses of secondary endpoints indicated that PBSCs may reduce the risk of graft failure, whereas BM may reduce the risk of chronic GVHD.

UCBs from unrelated donors can restore hematopoiesis in adults who receive myeloablative therapy and are associated with acceptable rates of severe acute and chronic GVHD. Apheresis products containing G-CSF-mobilized PBSCs are now widely used instead of BM for autologous transplantation. New drugs such as Mozobil (Genzyme) enhance mobilization of PBSCs, as shown in phase III clinical trials for non-Hodgkin lymphoma. Various stem cell transplantation techniques are shown in Fig. 10.1.

Types of cancer where stem cell transplantation has been found to be effective in combination with chemotherapy are:

- Acute lymphoblastic leukemia
- Acute myeloid leukemia (AML)
- Aplastic anemia
- Breast cancer
- Chronic myelogenous leukemia
- Hodgkin disease
- Lymphoma
- Malignant primary brain tumors
- Melanoma
- Multiple myeloma
- Ovarian cancer
- Small cell lung carcinoma
- Testicular cancer

Pharmaceuticals may be used to modulate the action of PBSCs. Valproic acid increases both proliferation and self-renewal of HSCs. It induces differentiation or apoptosis in leukemic blasts and stimulates the proliferation of normal HSCs. This effect has a potential future role in the treatment of acute myelogenous leukemia.

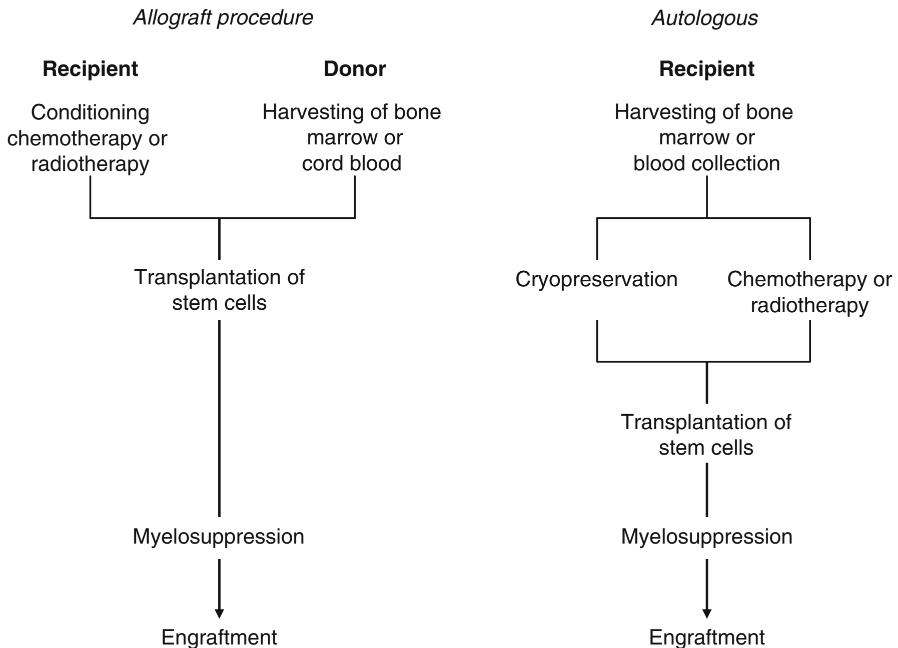

Fig. 10.1 Stem cell transplantation techniques. © Jain PharmaBiotech

10.5.2.2 Stem Cell Transplantation for Hematological Malignancies

Traditionally hematological malignancies have been treated with BM transplants. Now less than half of BM transplants are conducted the old way by harvesting marrow cells from the donor's hip. In more than half the cases, it involves drawing the donor's blood and separating stem cells by running it through a cell sorter. However, finding donors is challenging because the cells must be a near-perfect genetic match with the patient's own cells. Even siblings have compatible marrow cells only 30 % of the time. Most patients must search nationally and internationally for potential donors.

Acute myeloid leukemia (*AML*). A retrospective study that compared PBSCs to BM in matched unrelated donor transplantation for AML patients in remission showed that it resulted in the same rates of leukemia-free survival (Ringden et al. 2012). In patients with advanced leukemia, the PBSC group had reduced non-relapse mortality and improved leukemia-free survival.

Multiple myeloma. This is a malignancy of mature B cells. The disease is uniformly fatal, with a median survival of 3 years for patients receiving conventional chemotherapy. Death occurs within 1 year for most patients not receiving chemotherapy at all. High-dose chemotherapy followed by autologous stem cell transplantation has resulted in response rates ranging from 22 to 40 %. Patients with an initial response

to chemotherapy are more likely to respond to the high-dose chemotherapy/autologous stem cell regimens. Although autologous stem cell transplantation is yielding durable responses, allogeneic stem cell transplantation is the only potential cure for multiple myeloma. High-dose chemotherapy with autologous stem cell rescue is an effective first-line treatment for patients with multiple myeloma who are younger than 65 years of age.

The overall survival for myeloma patients receiving an allogeneic transplant is lower than in those receiving autologous transplant. This is primarily due to the advanced age of the patients and the increased serious transplantation-related toxicities associated with allogeneic transplant such as GVHD. There is improved survival with allogeneic transplantation patients who survive for more than 1 year, and this is possibly due to a graft-versus-myeloma effect.

Relapse of the disease is the primary cause of treatment failure following transplantation. Therefore, methods need to be developed to decrease the risk of relapse. Such methods may include purging the BM of residual myeloma cells, improving the transplantation procedure, or administering posttransplantation immunotherapy targeted at the residual tumor.

Lymphoma. The standard first-line chemotherapy in patients with lymphoma is a regimen of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP). High-dose chemotherapy in patients with aggressive disseminated lymphoma is supported by transplantation of autologous HSCs. In a randomized trial, high-dose chemotherapy with autologous HSC support was found to be superior to CHOP in adults with disseminated aggressive lymphoma as judged by the 5-year survival rate (Milpied et al. 2004). However, in another study of intensive chemotherapy with CHOP regimen followed by autologous stem cell transplantation in previously untreated patients with peripheral T-cell lymphoma, there was moderate complete remission rate (Mercadal et al. 2008). Contribution of autologous HSC transplant in preventing relapse is considered debatable.

Myelodysplastic syndrome (*MDS*). At present, allogeneic HSC transplantation is the only treatment that can induce long-term remission in patients with MDS. Such therapy, however, is not applicable for most patients, since the median age at diagnosis exceeds 70 years. HSC transplantation is associated with a high rate of treatment-related death (approximately 39 % at 1 year), suboptimal disease-free survival (approximately 29 % at 5 years), and chronic GVHD (~15 % at 1 year). The use of reduced-intensity conditioning regimens has been found to decrease the toxic effects of HSC transplantation but at the cost of an increased relapse rate. Therefore, allogeneic HSC transplantation is recommended only for patients with advanced-stage disease (Tefferi 2009).

10.5.2.3 Long-Term Results of HSC Transplantation

According to a study by researchers at Fred Hutchinson Research Cancer Center (Seattle, WA), survivors of HSC transplantation for hematological malignancies can

expect to be just about as healthy 10 years later as adults who have never had a transplant. Approximately 10 % of the survivors who had suffered relapse were in complete remission at the time of the study. In the past, relapse after a transplant was always considered to be a very bad sign for quality of life (QOL). The study found that transplant survivors and case-matched controls had similar rates of hospitalization and outpatient medical visits as well as similar QOL. However, transplant patients had a higher incidence of musculoskeletal problems, such as stiffness and cramping, poor long-term sexual health, and increased urinary frequency than the control group. Long-term survivors also had higher use rates of antidepressant and antianxiety medications even though reported rates of depression and anxiety were about the same as that of the control group. Underdiagnosed problems detected in survivors included osteoporosis and hypothyroidism. A study reveals that within 10 years of an allogeneic HSCT, the relative risk of a second, solid cancer is almost twice that of the general population (Gallagher and Forrest 2007). In addition, cancer risk almost quadruples for patients who were over 40 years old at the time of transplant or for patients who received stem cells from a female donor.

10.5.2.4 Prediction of T-Cell Reconstitution After HSC Transplantation

The extent and rapidity with which T cells are regenerated from graft-derived precursor cells directly influences the incidence of infection and the T-cell-based graft-versus-tumor (GVT) effect. Measurement of T-cell receptor excision circles (TRECs) in peripheral blood is a means of quantifying recent thymic T-cell production and has been used after HSC transplantation in many studies to estimate thymus-dependent T-cell reconstitution. In one study real-time PCR was used to quantify signal-joint TRECs (sjTRECs) before and after transplantation (Chen et al. 2005). The rate of reconstitution of thymus-dependent T cells was found to be dependent on the competence of thymic function in the recipients before transplantation. Therefore, pre-transplantation measurement of sjTREC may provide an important tool for predicting thymus-dependent T-cell reconstitution after transplantation.

Antigen-specific immune responses are impaired after allogeneic HSC transplantation. The events contributing to this impairment include host hematolymphoid ablation and donor cell regeneration, which is altered by pharmacologic immune suppression to prevent GVHD. A generally accepted concept is that graft T-cell depletion performed to avoid GVHD yields poorer immune recovery because mature donor T cells are thought to be the major mediators of protective immunity early post-HSCT. The findings of a study contradict the idea that removal of mature donor cells worsens immune recovery following HSC transplantation (Tsao et al. 2009). By transplantation of purified HSCs compared with BM across donor and recipient pairs of increasing genetic disparity, the investigators showed that grafts composed of the purified progenitor population give uniformly superior lymphoid reconstitution, both qualitatively and quantitatively. Subclinical GVHD by T cells in donor BM likely caused this lympho-depleting GVHD. They further determined in the MHC-mismatched pairs that T-cell-restricted proliferative responses were dictated by donor rather than host elements.

10.5.2.5 HSC Transplantation Followed by GM-CSF-Secreting Cell Vaccines

HSC transplantation (HSCT) provides durable clinical benefits for many patients with hematological malignancies through an immune-mediated graft-versus-leukemia (GvL) effect, but patients with high-risk AML often relapse, indicating the need for intensification of tumor immunity. In preclinical models, allogeneic HSCT followed by vaccination with irradiated tumor cells engineered to secrete GM-CSF generates a potent antitumor effect without exacerbating the toxicities of GVHD. In a phase I clinical trial, high-risk AML or myelodysplasia patients were immunized with irradiated, autologous, GM-CSF-secreting tumor cells early after allogeneic, nonmyeloablative HSCT (Ho et al. 2009). Vaccination elicited local and systemic reactions that were qualitatively similar to those previously observed in nontransplanted, immunized solid tumor patients. While the frequencies of acute and chronic GVHD were not increased, 9 of 10 subjects who completed vaccination achieved durable complete remissions. These results establish the safety and immunogenicity of irradiated, autologous, GM-CSF-secreting leukemia cell vaccines early after allogeneic HSCT and raise the possibility that this combined immunotherapy might potentiate graft-versus-leukemia in patients.

10.5.2.6 HSC Transplantation for Renal Cell Cancer

Patients with metastatic renal cell carcinoma (RCC) have been treated by nonmyeloablative allogeneic HCT for over a decade. However, during this period targeted therapies directed against VEGF or mammalian target of rapamycin (mTOR)-signaling pathways were introduced as first- and second-line treatments of RCC leading to uncertainty about a continued role for allogeneic HCT for RCC. A composite review of published reports, describing key efficacy and toxicity outcomes for 398 patients with metastatic RCC treated by allogeneic HCT, has been published (Tykodi et al. 2011). Correlative studies that identify donor-derived T cells as mediators of RCC-specific GVT effects offer insight into both the potential and the technical barriers to the delivery of antigen-specific posttransplant cellular therapy or vaccination designed to augment the allogeneic GVT effect. Moreover, serious adverse effects of allogeneic HCT were a handicap. Future development of allogeneic HCT for metastatic RCC needs novel treatment protocols designed to augment and sustain posttransplant GVT effects against RCC to renew enthusiasm for this approach.

10.5.3 Complications of Stem Cell Transplants in Cancer

10.5.3.1 Graft-Versus-Host Disease

GVHD is a common and potentially severe side effect of HSC transplants, in which donor immune cells attack normal patient cells and tissues. GVHD is more frequent in patients receiving transplants from mismatched-unrelated donors (in comparison

with matched-related donors). Steroids along with calcineurin inhibitors are the standard initial therapy, but less than half of the patients show adequate response. There is no uniformly accepted therapy for patients with steroid-resistant GVHD. Various MAbs to cell surface antigens on GVHD effector cells have been investigated for the treatment of acute GVHD: these include anti-TNF-α antibodies, IL-2 receptor antagonists, and anti-CD3 and anti-CD52 MAbs, while anti-CD20 mAb has been extensively investigated in the setting of chronic GVHD (Busca 2011). Overall, response rates have been reported to be >60 %, but long-term survival still remains suboptimal, mainly due to the infectious complications, progressive GVHD, and relapse of underlying malignancy. MAb combined with newer immunosuppressive drugs might achieve greater success, especially if used early in the disease process.

Bortezomib, as shown in preclinical models and in multiple myeloma patients who have received donor stem cell transplants, helps to control the overactivity of immune cells responsible for GVHD. Bortezomib has been shown to selectively reduce activity of an important nuclear factor-κB in T cells and prevent them from targeting patients' normal cells. Early administration of bortezomib protects against GVHD without reducing the transplanted stem cells' ability to settle in the BM. Clinical trials have shown that bortezomib shows promise in reducing GVHD and reconstituting immune system in stem cell transplant patients. The results show that the bortezomib-based therapy is safe and has little toxicity. Transplanted stem cells took root or engraft reliably, and the rate of GVHD in the bortezomib-based mismatched-unrelated transplants is comparable to that in sirolimus-based matched-related transplants.

10.5.3.2 Delayed Immune Reconstitution Leading to Viral Infections and Relapse

Graft rejection and the risk of GVHD can be significantly reduced by using intensive conditioning regimens, including in vivo T-cell depletion as well as ex vivo T-cell depletion of the graft. However, the benefits of removing alloreactive T cells from the graft are offset by the concomitant removal of T cells with antiviral or anticancer activity as well as the profound delay in endogenous T-cell recovery posttransplant. Thus, opportunistic infections, many of which are not amenable to conventional small molecule therapeutics, are frequent in these patients and are associated with significant morbidity and high mortality rates (Leen et al. 2010). Cell therapies have been used to prevent or treat viral infections/reactivations posttransplant.

10.5.3.3 Tumor Cell Contamination

One complication of using BM or peripheral blood as a source of stem cells in autologous (self-to-self) stem cell transplants is that the majority of these cell products also contain some tumor cells. By performing stem cell selection in autologous

transplantation, tumor cell contamination of the blood can be substantially reduced, thereby decreasing the risk of posttransplant disease recurrence associated with these tumor cells. Tumor cell contamination can be avoided if stem cells from a disease-free donor can be used. However, in this case, T cells from the donor can attack the cells of the recipient causing a severe life-threatening reaction known as GVHD. By performing stem cell selection of the donor's blood in allogeneic transplantation, T cells that can cause GVHD in the patient can be substantially reduced prior to the transplant.

The FDA-approved Isolex 300i cell selection system (Baxter) isolates and purifies stem cells from peripheral blood for use in stem cell transplantation to regenerate the blood or immune system following high-dose chemotherapy. Various studies have shown that automated CD34+ cell selection along with simultaneous purging with breast cancer antibodies or lymphoma antibodies can provide additional reduction of breast cancer or lymphoma tumor cells. Therefore, patients can be expected to receive substantially fewer tumor cells in their transplant product when this technology is utilized.

10.5.3.4 Neurological Complications

The frequency and cause of neurological complications following HSC transplantation were analyzed in 185 consecutive patients with malignant disease who underwent the procedure during a period of 5 years in a single institution (Batlle et al. 2005). After a median follow-up of 27 months, 14 patients (7.5 %) developed neurological complications. In this series drug toxicity was the main cause of neurological complications (particularly seizures) following HSC transplantation. These results differ from previous studies in which infections, especially of fungal origin, were the main cause of neurological complications.

A publication reports two patients who developed an acute myelitis within their thoracic spinal cord after allogeneic stem cell transplantation (Voss and Bischof 2010). Myelitis in these patients was not related to GVHD or immune reconstitution and was responsive to intravenous methylprednisolone and cyclophosphamide. Myelitis should be considered as a possibly disabling consequence of HSC transplantation. These cases also indicate that allogeneic HSCT carries the risk to induce autoimmune CNS disease with potentially disabling consequences. The incidence of myelitis after HSCT may be higher than previously estimated because similar cases may not have been reported and the incidence may vary between different ethnic groups.

10.5.3.5 Hepatic Veno-Occlusive Disease

Hepatic veno-occlusive disease (VOD) is a potentially life-threatening condition, which typically occurs as an important complication of HSC transplantation. Certain high-dose chemoradiation therapy regimens used as part of HSC transplantation can

damage the lining cells of hepatic blood vessels and so result in VOD, a blockage of the small veins of the liver that leads to liver failure and can result in significant dysfunction in other organs such as the kidneys and lungs (so-called severe VOD). HSC transplantation is a frequently used treatment modality following high-dose chemotherapy and radiation therapy for hematological cancers and other conditions in both adults and children. There is currently no approved agent for the treatment or prevention of VOD.

Defibrotide (Gentium SPA) is the sodium salt of a complex mixture of single-stranded oligodeoxyribonucleotides derived from porcine mucosal DNA. It has been shown that defibrotide has protective effects on vascular ECs, particularly those of small vessels. It has extensive beneficial pharmacological effects owing to its antithrombotic, anti-inflammatory, and anti-ischemic properties. Clinical trials of defibrotide for the treatment of severe hepatic VOD have indicated both efficacy and lack of significant toxicity. Other recent preclinical studies have demonstrated that defibrotide used in conjunction with rhG-CSF significantly increases the number of peripheral blood progenitor cell. In 2009, the results of a clinical trial demonstrated strong trends in favor of the defibrotide-treated patients for complete response and survival, but did not reach the protocol-specified levels of significance for the primary and secondary endpoints at 100 days. Further studies are anticipated.

10.5.3.6 Current Status of the Safety of Allogeneic HSC Transplantation

A study on a large number of patients has analyzed overall mortality, mortality not preceded by relapse, recurrent malignant conditions, and the frequency and severity of major complications of transplantation, including GVHD and hepatic, renal, pulmonary, and infectious complications (Gooley et al. 2010). The results showed a substantial reduction in the hazard of death related to allogeneic HSC transplantation, as well as increased long-term survival, over the past decade. Improved outcomes appear to be related to reductions in organ damage, infection, and severe acute GVHD. A study has shown that grafts composed of purified HSCs provide an optimal platform for in vivo expansion of selected antigen-specific cells while enabling the reconstitution of a naive T-cell pool (Müller et al. 2012). Pure HSC transplantations also allow superior protective immunity against a viral pathogen such as CMV compared with unselected mature T cells.

10.5.3.7 Complications of PBSC Transplantation in Children

Since PBSC has been shown to be as safe and effective as BM transplantation in adults, some physicians have begun recommending the use of PBSC transplantation in pediatric patients as well. It is estimated that nearly 30 % of transplants from sibling donors in pediatric patients now use PBSCs. However, transplantation of PBSC from sibling donors may be more harmful than BM in pediatric leukemia

patients. Chronic GVHD is significantly higher after PBSC transplants than after BM transplants. Patients undergoing PBSC transplants are more likely to die of transplant-related causes than those who undergo bone marrow transplantations (BMTs). These data support the use of BMT in these patients when a matched sibling donor is available. The higher rates of mortality observed after PBSC transplantation point out the need for a properly designed clinical trial to define the role, if any, of donor PBSC in children.

Severe neurological complications are frequent among children receiving PBSC transplants. Transplant from allogeneic donor, especially if unrelated, the development of severe acute GVHD grade >2, and the use of total body irradiation in the preparative regimen are the main risk factors for such complications.

According to a Finnish study, more than one third of children and adolescents who undergo allogeneic stem cell transplantation have diminished bone mineral density, and one in five have crushed vertebrae in their spines that are usually asymptomatic (Taskinen et al. 2007). The investigators conclude that because of the heightened risk of bone thinning and vertebral fractures, children undergoing stem cell transplantation should be carefully monitored after the procedure and possibly given drugs that improve osteoporosis.

10.5.4 Role of MSCs in Cancer

Human mesenchymal stem cells (hMSCs) give rise to marrow stromal cells that produce the spongy stromal matrix comprising the BM microenvironment. Nonhematopoietic MSCs produce the extracellular matrix where blood cell development takes place and provide cytokines, growth factors, as well as other molecules that direct or stimulate the production of mature blood cells. MSCs are capable of homing to the BM following intravenous infusion and have the capacity to establish residence within the BM for an extended duration following systemic administration. Infusion of autologous MSCs in breast cancer patients at the time of PBPC transplantation is feasible and safe. The observed rapid hematopoietic recovery suggests that MSC infusion after myeloablative therapy may have a positive impact on hematopoiesis. Clinical trials have evaluated safety and utility of hMSCs for autologous and allogeneic transplantation. Future studies in patients undergoing autologous transplantation are planned to include diseases such as lymphoma, myeloma, and leukemias.

10.5.4.1 MSC-Mediated Delivery of Anticancer Therapeutics

The ability of MSCs to specifically home to sites of tumors and their metastases, while escaping host immune surveillance, holds tremendous promise for tumor-targeted delivery of therapeutic agents. Specific anticancer gene-engineered MSCs have multiple-targeted potential as they migrate in vivo to tumors with

incorporation and in situ expression of tumor-specific anticancer genes providing personalized cancer therapy (Dai et al. 2013). Concerns that MSCs may have an inherent capacity for transformation have led to a number of studies to investigate their stability in vitro because significant ex vivo expansion will be necessary to yield the number of cells required for therapeutic applications. Tumors utilize many of the same inflammatory mediators uncovered in wound healing and likewise provide a site for preferential MSC homing. Although incorporation into the tumor microenvironment is apparent, the role of recruited MSC in the tumor microenvironment remains unclear. Some published studies have shown enhancement of tumor growth, perhaps through immunomodulatory and proangiogenic properties, while others have shown no apparent effect or have demonstrated inhibition of tumor growth and extended survival. This controversy remains as clinical applications of MSC have started in anticancer therapies as well as adjuncts to BMSC transplantation and in ameliorating GVHD. Careful analysis of past studies and thoughtful design of future experiments will help to resolve the discrepancies in the field and lead to clinical application of MSC in treatment of cancer. An understanding of interactions between MSCs and tumor cells is required to realize their clinical potential (Dwyer and Kerin 2010).

10.5.5 Nonmyeloablative Allogeneic Hematopoietic Stem Cell Transplantation

During the past few years, there has been an explosion of knowledge in nonablative allogeneic stem cell transplantation. The principle governing this approach is the occurrence of an allogeneic GVT effect. It relies more on the creation of "immunological space" for engraftment rather than the more traditional approach of creating "physical space" by the application of either intensive radiation or chemical therapy. The treatment first suppresses the patient's own immune system with drugs so that compatible sibling stem cells can be transfused. Gradually, the donor cells replace the patient's immune system and stem cells; the transplanted immune system then attacks and kills the tumor cells.

The strategy of allogeneic transplantation after nonmyeloablative conditioning has been proposed to patients who meet the following conditions: (1) have either lymphomas, chronic myeloid leukemia, or myeloma; (2) receive a large number of CD34+ (selected) cells; (3) are treated after a regimen containing fludarabine and a long GVHD prophylaxis followed by prophylactic and preemptive donor lymphocyte infusion (DLI); and (4) have documented chimerism and minimal residual disease. The optimum program for each disease, the best HSC source, GVHD prophylaxis adjustment, and donor–recipient pairing have yet to be defined.

Nonablative allogeneic stem cell transplantation holds the promise of allowing powerful alloimmune responses to eradicate disease processes while minimizing the initial treatment-related morbidity and mortality, and it appears to be the necessary enabling platform by which to apply allogeneic cellular therapy. This approach

should broaden the eligibility for potentially curative allogeneic transplantation in various diseases, reduce initial hospitalization costs, and at the same time have a positive impact on QOL.

Mixed-chimerism bone marrow transplantation (mini-BMT) is another term used for nonmyeloablative allogeneic transplantation. The goal of a mini-BMT is to immunosuppress the patient to facilitate stem cell engraftment. The process produces a mixed chimeric state that may enable the delivery of an antileukemic effect through DLI. There is evidence to suggest that a graft-versus-leukemia effect plays a role in the improved response rates seen with allogeneic transplantation over autologous transplantation. There is an inverse relationship between relapse and GVHD.

Patient selection is important when considering a mini-BMT. Patients with rapidly progressive disease may not benefit from a mini-BMT, since the effects of the transplantation rely upon a GVHD effect, which is delayed. The disease states that have been studied include leukemia, MDS, lymphoma, multiple myeloma, RCC, malignant melanoma, breast cancer, and aplastic anemia. Multiple chemotherapy agents have been used, including fludarabine, cladribine, pentostatin, antithymocyte globulin, cyclophosphamide, sublethal total body irradiation, and Campath 1H (an investigational anti-CD52 monoclonal antibody).

The incidence of GVHD with mini-BMT is roughly 35–50 %, but the transplantation-related mortality at 1 year is less than 10 %, which is greatly improved over conventional transplantation. At this time, mini-BMT is still under investigation. The long-term results are not known and the role of DLI is unclear.

10.5.6 Umbilical Cord Blood Transplant for Leukemia

Umbilical cord blood (UCB) transplantation, used for patients with leukemia, is less likely to produce a reaction in spite of differences between patient and donor, making it possible to perform this procedure without an exact match. Although stem cell transplantation using UCB is a standard treatment option for blood disorders in children, it does not apply to adults due to the difficulty of obtaining a sufficiently large dose of cells. To solve this problem, a technique that combines two partially HLA-matched UCB units from different donors has been found to be safe for transplantation into adult or adolescent leukemia patients. With this technique of increasing the dose by combining two units, this procedure could be made more widely available to patients.

A study has directly compared matched BM, which is currently considered the preferred graft, with matched and mismatched UCB in patients with leukemia. The results showed that mismatched UCB performed as well as matched BM as measured by leukemia-free survival rates, providing the degree of mismatch was limited and the number of UCB cells available was sufficient (Eapen et al. 2007). Study participants who received matched UCB had a 20 % higher survival rate than matched BM recipients, though the number of matched UCB transplants was small. Thus, UCB transplants may offer blood cancer patients better outcomes than BM transplants.

Scientists at M.D. Anderson's Department of Stem Cell Transplantation and Cellular Therapy have found a novel process to increase NK cells in UCB more than 30-fold, generating more than 150 million NK cells from one UCB unit while maintaining their activation to find and kill acute leukemia cells. When given to mice with aggressive human leukemias, these NK cells reduced the circulating human acute lymphocytic leukemia (ALL) and acute myelogenous leukemia (AML) cells by 60–85 %. As the cord blood is expanded to multiply in number, the NK cells are given a cytokine, interleukin-2, and a target cell, K562, which keep the NK cells active throughout the 3-week expansion. Once the process is complete, the NK cells can be transplanted to patients without prior chemotherapy. This could be used for patients who are not candidates for other stem cell transplants due to blood counts or illness.

10.5.7 hESC-Derived NK Cells for Treatment of Cancer

hESCs provide a unique resource to analyze early stages of human hematopoiesis. A two-step culture method can demonstrate efficient generation of functional mature NK cells from hESCs. The hESC-derived lymphocytes express inhibitory and activating receptors typical of mature NK cells, including killer cell Ig-like receptors, natural cytotoxicity receptors, and CD16. The hESC-derived NK cells acquire the ability to lyse human tumor cells by both direct cell-mediated cytotoxicity and Ab-dependent cellular cytotoxicity. Additionally, activated hESC-derived NK cells upregulate cytokine production. hESC-derived lymphoid progenitors provide a novel means to characterize specific cellular and molecular mechanisms that lead to development of specific human lymphocyte populations. These cells may also provide a source for innovative cellular immune therapies for cancer. This study showed the ability to make cells from hESCs that are able to treat and fight cancer, especially leukemias and lymphomas.

10.5.8 ESC Vaccine for Prevention of Lung Cancer

Scientists at the James Graham Brown Cancer Center (Louisville, KY) have discovered that vaccinating mice with ESCs prevented lung cancer in those animals that had cancer cells transplanted into them after the vaccination or that had been exposed to cancer-causing chemicals. Two types of vaccines have been tried: (1) consisting of ESCs only, obtained from mouse blastocysts, and (2) ESCs combined with cultured fibroblast cells producing GM-CSF immunity to cancer. Tumors arising in vaccinated mice were, on average, about 80–90 % smaller than tumors in unvaccinated mice. All the unvaccinated mice developed tumors. None of the vaccinated mice developed autoimmune disease or showed a significant decline in adult pluripotent BM stem cells—both potential adverse responses to the vaccinations.

The findings suggest that it could be possible to develop ESC vaccines that prevent cancers in humans, such as hereditary breast and colon cancer and lung cancer caused by smoking or other environmental factors.

10.5.9 Genetic Modification of Stem Cells for Cancer Therapy

10.5.9.1 Genetic Modification of Hematopoietic Stem Cells

Gene transfer into human HSCs enables enhancement of anticancer immunity as well as increases their resistance to cytotoxic drugs. MDR (multiple drug resistance) gene can be introduced in HSC to reduce chemotoxicity and allow administration of higher doses of chemotherapy. Tumor cell eradication can also be enhanced by genetic modification of chemosensitivity and immunomodulation.

Hematopoietic cells differentiate in steps marked by the acquisition or loss of specific phenotypic characteristics. It is possible to efficiently purify cells with characteristics of HSCs by using techniques combining cytokine stimulation with antimetabolic treatments. Cytokines have also been shown to improve gene transfer to human HSCs.

Gene marking of hematopoietic cells provides no direct therapeutic advantage to patients, but information gained from these studies helps to improve the outcome of therapies that incorporate autologous HSC transplantation as a device for eradicating tumors. Several clinical studies have used retroviral gene-marked autologous blood or BM cells to examine the engraftment of hematopoietic cells following high-dose chemotherapy and to determine the contribution of tumor cells contaminating the autograft to relapse of the disease. Double gene marking has been used to monitor BM purging and to compare the long-term reconstitution from different populations of hematopoietic progenitor cells.

10.5.9.2 Delivery of Anticancer Agents by Genetically Engineered MSCs

MSCs have unique properties, which make them ideally suited as vehicles for gene and drug delivery to treat cancer. These properties include the following: (1) relative ease of isolation, (2) ability to be expanded in culture without loss of their ability to differentiate, (3) hypoimmunogenicity, and (4) ability to home to tumors and metastases following in vivo administration. The use of MSCs as carriers of anticancer agents is limited by the toxic effect of these agents on cells, and to overcome this, MSCs have been genetically engineered to produce antitumor proteins. An example of this is as follows.

High concentrations of interferon-β (IFN-β) inhibit malignant cell growth in vitro. However, the therapeutic utility of IFN-β in vivo is limited by its excessive toxicity when administered systemically at high doses. Human MSCs, transduced with an adenoviral expression vector carrying the human IFN-β gene, can deliver

IFN-β to tumors, reducing toxicity. Injected MSC-IFN-β cells suppress the growth of pulmonary metastases, presumably through the local production of IFN-β in the tumor microenvironment. Therefore, MSCs may act as "cellular vehicles" that pump drugs directly into cancer cells to disable them, but leave normal tissue alone.

What facilitates this approach is the nature of tumors as chronic nonhealing wounds, which signal these stem cells and then use them to help build up stromal or connective tissue for structural and nutritional support of tumor growth. Tumors constantly remodel their architecture with the help of these special stem cells. This method would work particularly well after patients are treated with radiation or chemotherapy, because those therapies damage cancer cells, which would then be in critical need of MSCs. This homing strategy might offer a novel way to treat cancer that has spread. This drug delivery system is attracted to cancer cells no matter what form they are in or where they are.

Suicide gene introduction into human adipose tissue (AT)-MSC has the potential to produce a tumor-specific prodrug-converting cellular vehicle for targeted chemotherapy. Their therapeutic potential has been explored in a model of human colon cancer in the presence of prodrug 5-fluorocytosine (5-FC). Gene manipulation of human AT-MSC did not sensitize CD-AT-MSC to 5-FC, thus overcoming the inherent disadvantage of suicide effect on cellular vehicle (Kucerova et al. 2007). CD-AT-MSC in combination with 5-FC augmented the bystander effect and selective cytotoxicity on target tumor cells in direct coculture in vitro. We confirmed directed migration ability of AT-MSC and CD-AT-MSC toward tumor cells HT-29 in vitro. Furthermore, significant inhibition of subcutaneous tumor xenograft growth was achieved by intravenously administered CD-AT-MSC in immunocompromised mice treated with 5-FC. The ability of CD-AT-MSC to deliver the CD transgene to the site of tumor formation and mediate strong antitumor effect in vivo was confirmed. These data characterize MSC derived from adipose tissue as suitable delivery vehicles for prodrug-converting gene and show their utility for a personalized cell-based targeted cancer gene therapy.

10.5.9.3 Mesenchymal Progenitor Cells for Delivery of Oncolytic Adenoviruses

Natural and genetically modified oncolytic viruses have been systematically tested as anticancer therapeutics. Among this group, conditionally replicative adenoviruses have been developed for a broad range of tumors with a rapid transition to clinical settings. However, clinical trials have shown limited antitumor efficacy partly due to insufficient viral delivery to tumor sites. The use of mesenchymal progenitor cells (MPCs) as virus carriers is based on the tumor-homing abilities of this cell population. The preferential tumor homing of MPCs was confirmed in an animal model of ovarian carcinoma and demonstrated to be efficiently infected with a genetically modified adenovirus Ad5/3 (Komarova et al. 2006). MPCs loaded with Ad5/3 caused total cell destruction when cocultured with a cancer cell line. In an

animal model of ovarian cancer, MPC-based delivery of the Ad5/3 increased the survival of tumor-bearing mice compared with direct viral injection. Further, tumor imaging confirmed a decrease in tumor burden in animals treated with oncolytic virus delivered by MPC carriers compared with the direct injection of the adenovirus. These data show that MPCs can serve as intermediate carriers for replicative adenoviruses and suggest that the natural homing properties of specific cell types can be used for targeted delivery of these virions.

10.5.9.4 Genetically Modified NSCs for Treatment of Neuroblastoma

Neuroblastoma is the most common solid tumor in children that arises in the part of the nervous system outside the brain. Typically, patients diagnosed with high-risk disease demonstrate a good initial response to therapy, but as many as 80 % of these patients relapse with metastatic disease that is refractory to therapy. Like other solid tumors, when neuroblastomas metastasize, they are very difficult to treat, and a majority of children with metastatic neuroblastoma die of their disease. Clinicians are limited in how aggressively they can treat these children because the chemotherapy drugs produce severe side effects and therefore must be administered at reduced levels. There is a need for new treatments for neuroblastoma that target tumor cells while having minimal side effects.

Mice with neuroblastoma tumors have been successfully treated with genetically modified neural stem cells (NSCs) that sought out the cancer cells and activated a chemotherapy drug directly at those sites (Aboody et al. 2006). The approach described in this study is based on exploiting the tumor-tropic property of HB1.F3.C1 cells derived from NSCs and immortalized by retroviral insertion of the v-myc gene, which was required to sustain their replication potential. The specific goal of the study was to show that intravenous administration of HB1.F3.C1 cells expressing the CPT-11 (irinotecan)-activating enzyme rabbit carboxylesterase (rCE) would significantly increase the antitumor effect of tolerated doses of CPT-11 in mice bearing disseminated neuroblastoma tumors. The drug dispersed throughout the mice but was activated by rCE selectively at the site of neuroblastoma tumors. This activation is essential to treatment because the activated form is up to 1,000 times more active than CPT-11. The researchers also showed that the modified cells migrated to tumors regardless of how small the tumors were or where they were located in the body. The study is the first to provide evidence that NSCs can be used to target solid tumors that have metastasized. This homing ability is especially important in the case of high-risk neuroblastoma because even very small tumors that survive after an initially successful treatment often generate more cancer cells that spread and become unresponsive to treatment. The ability to target tumors with CPT-11 suggests that this technique could let clinicians treat tumors in humans more effectively while avoiding side effects caused by damage to normal cells. The success with neuroblastoma also suggests this technique might improve the treatment of other solid tumors that metastasize, such as colon and prostate cancer.

10.6 Innovations in Cell-Based Therapy of Cancer

10.6.1 Use of Immortalized Cells

The maintenance of specialized nucleoprotein structures termed telomeres is essential for chromosome stability. Without new synthesis of telomeres at chromosome ends, the chromosomes shorten with progressive cell division, eventually triggering either replicative senescence or apoptosis when telomere length becomes critically short. The regulation of telomerase activity in human cells plays a significant role in the development of cancer. Understanding telomerase biology will eventually lead to several clinically relevant telomerase-based therapies. These applications include inhibiting or targeting telomerase as a novel antineoplastic strategy and using cells immortalized by telomerase for therapeutic applications.

10.6.2 Cancer Therapy Based on Natural Killer Cells

The use of NK cells in adoptive therapy for malignant disease is an area of great potential. NK cells mediate cell contact-dependent cellular cytotoxicity and produce proinflammatory cytokines, but do not rearrange antigen receptors. Their activation depends on various germline-encoded receptors, including CD16, which mediates recognition of antibody-coated target cells (Arina et al. 2007). NK cytotoxicity is checked by a repertoire of inhibitory receptors that scan adequate expression of major histocompatibility complex class I molecules on the potential target cell. Functional cross talk of NK and dendritic cells suggests a critical role for NK cells in the initiation and regulation of cellular immunity. The clinical use of NK cells in patients has undergone 15 years of refinement. Considerable knowledge on the molecular basis of NK recognition/activation contrasts with a lack of successful translational research on these matters. However, there is plenty of opportunity for targeted intervention of inhibitory/activatory surface receptors and for adoptive cell therapy with autologous or allogeneic NK cells. Other approaches in clinical evaluation include targeting heat shock protein (HSP) 70-expressing tumors with pre-stimulated autologous NK cells or the application of an NK cell line, NK-92, with enhanced cytolytic activity. Efficacy of NK-92 as a purging agent to decrease or eliminate malignant contamination of autologous stem cell grafts for chronic myeloid leukemia has been demonstrated. NK-92 has been tested in clinical trials, but there is no clinical development currently. It appears that genetically modified NK-92 cells are more effective than parent cells as anticancer agents.

10.6.3 Cytokine-Induced Killer Cells

In recent years, considerable progress in cancer treatment has been obtained by the application of cytokine-induced killer (CIK) cells. Application of CIK cells as

adoptive immunotherapy plays an important role in cancer treatment. Combining CIK cells with other conventional and established therapy options represents an innovative approach and will hopefully provide new insight for the future (Thanendrarajan et al. 2012). One clinical study has already shown that CIK cell transfusion therapy used in combination with gemcitabine and cisplatin chemotherapy may be a more effective treatment for postradiotherapy distant metastasis of nasopharyngeal carcinoma patients (Lian et al. 2012).

10.6.4 Nanomagnets for Targeted Cell-Based Cancer Gene Therapy

Using human cells as delivery vehicles for anticancer gene therapy is a promising approach for treating cancer. Monocytes naturally migrate from the bloodstream into tumors, and attempts have been made to use them to deliver therapeutic genes to these sites. However, transfected monocytes injected systemically fail to infiltrate tumors in large numbers. Therefore, nanoscale magnets have been developed to target cancer tumor cells more effectively (Muthana et al. 2008). The impact of gene therapy on cancer cells can be enhanced by "magnetic targeting," i.e., inserting nanomagnets into cells carrying genes so that the number of cells successfully reaching and invading cancer can be increased. Systemic administration of such "magnetic" monocytes to mice bearing solid tumors led to a marked increase in their extravasation into the tumor in the presence of an external magnet. Further studies are exploring the effectiveness of magnetic targeting in delivering a variety of cancer-fighting genes, including ones that could stop the spread of tumors. This technique could also be used to help deliver therapeutic genes in other diseases like arthritic joints or ischemic heart tissue.

10.6.5 Antiangiogenesis Therapy by Implantation of Microencapsulated Cells

Inhibition of angiogenesis is known to suppress tumors in several cancer models. Although administering purified recombinant antiangiogenic product is effective, alternative approaches through genetic manipulation may be more cost-effective. Nonautologous genetically modified cells secreting angiostatin have been implanted for systemic delivery of angiostatin in mice bearing solid tumors. These cells are protected from graft rejection in alginate microcapsules to function as "micro-organs" for delivery of angiostatin in vivo resulting in localization of angiostatin to tumors and targeted apoptosis of the ECs. Efficacy has been demonstrated by suppression of palpable tumor growth and improved survival.

Endostatin is a potent inhibitor of angiogenesis and tumor growth. Continuous delivery of endostatin improves the efficacy and potency of the antitumoral therapy.

Recombinant fibroblasts expressing endostatin encapsulated in TheraCyte immunoisolation devices were shown to be effective for the inhibition of the growth of melanoma and Ehrlich tumor-bearing mice (Rodrigues et al. 2010).

10.6.6 A Device for Filtering Cancer and Stem Cells in the Blood

A device called CellSelect (CellTraffic Inc.) filters the blood for cancer and stem cells. The captured cancer cells are killed but the stem cells are spared. The technique can be used in vivo, i.e., a device is inserted in the body, or in vitro, in which case the device resides outside of the body—either way, the device kills cancer cells and captures stem cells, which grow into blood cells, bone, cartilage, and fat. The underlying mechanism for this phenomenon is based on selectins, which stick onto specific carbohydrate receptors on the surfaces of white blood cells, stem cells, and cancer cells. The device, using a combination of microfluidic and specialized selectin coatings, captures stem and cancer cells before the selectins release them. Current procedures enable the specific capture of hematopoietic stem and progenitor cells, which differentiate into all of the different blood cells, and the specific capture of stem cells that differentiate into BM cells. In an experimental study, selectin-mediated capture of CD34+ stem cells was shown to result in enrichment that was ~8-fold higher than the CD34+ cell population from BM mononuclear cells (Narasipura et al. 2008). This study supports the hypothesis that flow-based, adhesion molecule-mediated capture may be a viable alternative approach to the capture and purification of stem cells.

A potential application of the device is filtering the blood for cancer cells and triggering their death. As a cancer cell flows along the implanted surface, the device captures it and delivers an apoptosis signal, telling the cancer cell to kill itself. Within 2 days, that cancer cell is dead. Normal cells are left totally unharmed because the device selectively targets cancer cells. The apoptosis signal is delivered by a molecule called TRAIL that coats the cancer-killing device. Cancer cells have proteins that recognize and bind to TRAIL, but healthy cells do not, giving them a natural protection against TRAIL. A possible way to use the cancer-killing device is to implant the device in the body before primary tumor surgery or chemotherapy. The device would capture cancer cells released into the bloodstream as a result of cutting the tumor and kill them to reduce the possibility of metastasis.

10.6.7 Cancer Stem Cells

Cancers may rely on cancer stem cells (CSCs) that share the self-renewal feature of normal stem cells. This has changed the perspective with regard to new approaches for treating cancer. ESCs rely on Polycomb group proteins to reversibly repress

genes required for differentiation. Stem cell Polycomb group targets are up to 12-fold more likely to have cancer-specific promoter DNA hypermethylation than nontargets, supporting a stem cell origin of cancer in which reversible gene repression is replaced by permanent silencing, locking the cell into a perpetual state of self-renewal and thereby predisposing to subsequent malignant transformation (Widschwendter et al. 2007).

CSCs have been investigated in several cancers including those involving the breast, prostate, pancreas, and intestine. Several signaling pathways are active in normal cells and CSC in these organs and provide opportunities for development of targeted therapies.

10.6.7.1 Role of Integrative Nuclear Signaling in Stem Cell Development

A novel gene signaling mechanism, integrative nuclear fibroblast growth factor receptor-1 (FGFR1) signaling (INFS), has been identified that controls whether a stem cell develops into its destined tissue or fails to differentiate and becomes cancer (Stachowiak et al. 2007). Activation of cell surface neurotransmitter, hormonal, or growth factor receptors stimulates the release of FGFR1 from cytoplasmic membranes into the cytosol. This process is enabled by the atypical transmembrane domain of FGFR1 and is facilitated by the interaction with pp90 ribosomal S6 kinase-1. Cytosolic FGFR1 is transported into the nucleus by importin beta and activates transcription in cooperation with CBP (cyclic AMP responsive element-binding protein) by augmenting RNA polymerase II activity and histone acetylation. To explain the developmental function of FGFR1, a “feed-forward-and-gate” signaling mechanism has been presented in which the INFS pathway “feeds forward” the developmental signals to the common and essential transcriptional coactivator, CBP. The coupled activation of CBP (by INFS) and transcription factors (by specific signaling pathways) enables the coordinated regulation of multigene programs by developmental cues. In some cancer cells, in which INFS is inactive, the reconstitution of nuclear FGFR1 signaling may be used to reestablish this coordinated regulation thereby inhibiting tumor cell proliferation and inducing differentiation. This mechanism presents a new and promising target for in vivo NSC therapies and anticancer strategies.

10.6.7.2 Cancer Stem Cell Markers

Several CSC markers have been identified but how they function is not well understood. Some of the markers frequently used to identify adult stem cells (ASCs) within the prostate, breast, and intestine are relevant to CSCs. Examples are as follows:

- CD24, a heat-stable antigen, is found in cell surface proteins on human mammary repopulating units in mice as well as on breast CSCs.
- ESA (epithelial specific antigen), a cell adhesion molecule, is found on breast and pancreatic CSCs; its expression level is elevated during carcinogenesis.

- CD49f (integrin α6) is a marker for coordination between cytoskeleton and adhesion to extracellular matrix and is found on prostate CSCs.
- CD34, a marker for liver and pancreas stem cells, is found on intestinal CSCs.

There is a need for better characterization of the biological function of known CSC markers. The discovery of additional CSC markers would enable more specific and targeted anticancer strategies that spare the normal tissues.

10.6.7.3 Breast Cancer Stem Cells

The presence of disseminated tumor cells in the BM of breast cancer patients is an acknowledged independent prognostic factor. Stem cells are a type of cell in breast tumors that are believed to seed the growth of new cancers. These cells are only a small part of the vast number of cells within tumors, but they can act like ASCs—a basic cell that can grow into different types of specialized cells. Much current research has focused on the theory that it is these stem cells landing in a distant site that creates metastases and not simply single cells that detach from the primary tumor and travel to another part of the body. Putative breast CSCs have been shown in the BM of early breast cancer patients, suggesting the risk of disease spread for all breast cancer patients may be greater than previously thought. The authors, who anticipated some stem cells within the disseminated tumor cells, were surprised that the majority of the remote tumor cells have the stem cell characteristics and that they appeared in the BM of breast cancer patients whose disease was caught in the earliest stages. This data suggest that the vast majority of patients with disseminated tumor cells may have a lifetime risk for relapse. Future molecular characterization of these cells is warranted.

The metastatic spread of epithelial cancer cells from the primary tumor to distant organs mimics the cell migrations that occur during embryogenesis. Gene expression profiling has revealed that the Mesenchyme Forkhead 1 (FOXC2) transcription factor, which is involved in specifying MSC fate during embryogenesis, is associated with the metastatic capabilities of cancer cells (Mani et al. 2007). Expression of FOXC2 is significantly correlated with the highly aggressive basal-like subtype of human breast cancers.

The induction of an epithelial–mesenchymal transition (EMT) in immortalized human mammary epithelial cells (HMEC) results in the acquisition of mesenchymal traits and in the expression of stem cell biomarkers. These cells have an increased ability to form mammospheres, a property associated with mammary epithelial stem cells. Independent of this, stem cell-like cells isolated from HMEC cultures form mammospheres and express biomarkers similar to those of HMECs that have undergone an EMT. Moreover, stem-like cells isolated from either mouse or human mammary glands or mammary carcinomas express EMT biomarkers. Finally, transformed human mammary epithelial cells that have undergone an EMT form mammospheres, soft agar colonies, and tumors more efficiently. These findings illustrate a direct link between the EMT and the gain of epithelial stem cell properties.

Thus, CSCs that are induced to follow one of these pathways may gain properties of ASCs, including the ability to self-renew and therefore their tumor-initiating ability. These findings have implications for regenerative medicine and for cancer treatment.

10.6.7.4 Role of Endothelial Progenitor Cells in Tumor Angiogenesis

Tumors build vessels by cooption of preexisting vasculature and de novo recruitment of BM-derived endothelial progenitor cells (EPCs). The precise spatial and temporal contribution of EPCs to the neovascularization of breast tumor has been demonstrated in vivo using high-resolution microscopy and flow cytometry (Nolan et al. 2007). Early tumors recruit BM-derived EPCs that differentiate into mature BM-derived ECs and incorporate into the lumens of a subset of sprouting tumor neovessels. Notably, in later tumors, these BM-derived vessels are diluted with non-BM-derived vessels from the periphery, which explain differences in previous reports on this topic. The results of this investigation show that EPCs are only present in the earliest stages of tumor progression, before the formation of blood vessels. Furthermore, specific ablation of BM-derived EPCs with α-particle-emitting anti-VE-cadherin antibody markedly impairs tumor growth associated with reduced vascularization. These results demonstrate that BM-derived EPCs play a critical role in the early stages of tumor progression and that eliminating EPCs should stop growth of cancer.

10.6.7.5 Role of Cancer Stem Cells in Metastases

Human pancreatic cancer tissue has been shown to contain CSCs defined by CD133 expression, which are exclusively tumorigenic and highly resistant to standard chemotherapy (Hermann et al. 2007). In the invasive front of pancreatic tumors, a distinct subpopulation of CD133+ CXCR4+ CSCs determines the metastatic phenotype of the individual tumor. Depletion of the CSC pool for these migrating CSCs virtually abrogates the metastatic phenotype of pancreatic tumors without affecting their tumorigenic potential. These findings demonstrate that a subpopulation of migrating CD133+ CXCR4+ CSCs is essential for tumor metastasis. Strategies aimed at modulating the SDF-1/CXCR4 axis may have important clinical applications to inhibit metastasis of CSCs. Although the study focused on pancreatic cancer, it could apply to many other tumors as well.

10.6.7.6 Therapeutic Implications of Cancer Stem Cells

CSCs form new tumors and may not be eliminated by current therapies. One difference between normal stem cells and CSCs is their degree of dependence on the stem cell niche, a specialized microenvironment in which stem cells reside. The stem cell niche in adult somatic tissues plays an essential role in maintaining stem cells or

preventing carcinogenesis by providing primarily inhibitory signals for both proliferation and differentiation. However, the niche also provides transient signals for stem cell division to support ongoing tissue regeneration. The balance between proliferation-inhibiting and proliferation-promoting signals is the key to homeostatic regulation of stem cell maintenance versus tissue regeneration. Loss of the niche can lead to loss of stem cells, indicating the reliance of stem cells on niche signals. Therefore, CSCs may arise from an intrinsic mutation, leading to self-sufficient cell proliferation. Furthermore, the molecular machinery used by normal stem cells for homing to or mobilizing from the niche may be "hijacked" by CSCs for invasion and metastasis. An understanding of interaction between stem cells and their niche will provide an insight into the process of cancer development, invasiveness, and metastasis. This may reveal potential new targets for cancer therapy.

The cells under continuous exogenous and endogenous genotoxic stress accumulate DNA errors, drive proliferative expansion, and may transform into CSCs with a heterogeneous population of tumor cells. These cells are a common phenomenon for the hematological malignancies and solid tumors. In response to DNA damage, the complex cellular mechanisms including cell cycle arrest, transcription induction, and DNA repair are activated. Exposure to cytotoxic agents should lead to cell death, but the absence of repair machinery makes the cells resistant to tumor-sensitizing agents and results in malignant transformation. Mismatch repair (MMR) gene defects have been identified in hematopoietic malignancies, leukemia and lymphoma cell lines. MMR systems are important in maintaining the stem cell functioning and have therapeutic implications in the eradication of CSCs and differentiated tumor cells (Vaish 2007). An understanding of the biological functions of MMR in the stem cells and its malignant counterparts could help in developing effective novel therapies for cancer.

It is generally assumed that normal and neoplastic stem cells differentiate into nonstem progeny in a unidirectional manner; a subpopulation of basal-like human mammary epithelial cells has been identified that departs from that assumption as they spontaneously dedifferentiate into stem-like cells (Chaffer et al. 2011). Such plasticity may enable derivation of patient-specific ASCs without genetic manipulation and has important implications for therapeutic strategies to eradicate cancer.

The current development of cancer therapeutics based on tumor regression may have produced agents that kill differentiated tumor cells while sparing the rare CSC population. Cancer treatments must target CSCs to eradicate the disease. OncoMed Pharmaceuticals' lead anti-CSC therapeutic, demcizumab, is a MAb optimized to block delta-like ligand 4 (DLL4), an activator of notch signaling, which is a pathway known to be important in CSCs and cancer. Blocking of DLL4 results in broad-spectrum anticancer activity via multiple mechanisms that include disruption of angiogenesis, inhibition of CSC growth, and promotion of cell differentiation. It is in phase Ib combination studies with standard chemotherapy in advanced NSCLC and pancreatic cancers. Other approaches include the following: (1) training immune cells to recognize and attack CSCs, (2) the use of existing drugs to alter signaling in order to deprive CSCs of environmental clues that help them to thrive, and (3) developing drugs to force CSCs to differentiate, which would take away their ability for self-renewal.

10.6.7.7 Targeting Cancer Stem Cells in Leukemia

The progression of some cancers, including leukemia, appears to be driven by CSCs. To have any hope of curing cancer, it is necessary to develop therapies that kill CSCs. Yet these cells frequently have properties that are similar to normal stem cells. This raises the question of whether disease therapies can be developed that eliminate CSCs without eliminating normal stem cells. This issue was addressed by conditionally deleting the PTEN tumor suppressor gene in adult hematopoietic cells, which led to myeloproliferative disease within days and transplantable leukemias within weeks (Yilmaz et al. 2006). PTEN deletion also promoted HSC proliferation, but in contrast to leukemia-initiating cells, HSCs were therefore unable to maintain themselves without PTEN. These effects were mostly mediated by mTOR as they were inhibited by rapamycin, a drug that reduces the activity of this metabolic pathway and is currently being tested in clinical trials for activity against a variety of cancers. Rapamycin not only depleted leukemia-initiating cells but also restored normal HSC function. Mice that were given rapamycin immediately after PTEN deletion failed to develop leukemia. Mice that already had leukemia were kept alive longer by the drug. Differences between normal stem cells and CSCs can be exploited to deplete CSCs without damaging normal stem cells in the same tissue. Thus, it will be possible to develop new anticancer therapies that are more effective and less toxic. This is important for leukemia patients that often cannot be cured with current therapies and for whom existing therapies sometimes have fatal side effects. However, the study raises the possibility that rapamycin could be effective in depleting CSCs in leukemia for at least certain patients. Clinical trials are needed to test this in human patients.

A mathematical model, based on stem cell population as a birth–death process, predicts how selective a therapy must be to ensure that enough HSCs survive when CSCs have been eradicated (Sehl et al. 2009). This enables comparison of CSC and HSC eradication times under therapy and calculation of the number of HSCs at the time of CSC eradication for varied initial population sizes and stem cell death rates. From a clinical point of view, these models provide criteria for assessing safety of CSC therapy. In conjunction with experimental observation of CSC killing rates, these results will be useful in screening targeted therapies for both hematological malignancies and solid tumors.

10.6.7.8 Targeting CSCs in Ovarian Cancer

Although epithelial ovarian cancer cells are eliminated by debulking surgery and chemotherapy during initial treatment, it is believed that only a subset of cancer cells, CSCs, may be an important source of tumor recurrence and drug resistance. Using mouse ovarian cancer models, a study has highlighted the role of CSCs in response to chemotherapy, their impact on recurrence (Ahmed et al. 2013). Understanding the distinct mechanisms that facilitate CSC survival and propagation is likely to reveal opportunities for improving the treatment outcomes for ovarian cancer patients. Knowledge of relationship between epithelial-to-mesenchymal

transition and CSC might facilitate development of strategies to induce cancer cells to differentiate into benign stromal fibroblasts in response to certain chemotherapy drugs (Chen et al. 2013). In the future, individualized therapy must incorporate analysis of the CSC subpopulation of ovarian cancer cells when designing therapeutic strategies for ovarian cancer patients.

10.7 Future of Cell-Based Immunotherapy for Cancer

Most clinical trials to date have vaccinated patients with advanced disease. These patients will have some degree of immunosuppression, from the cancer itself and as a result of previous treatment. Immunization strategies are likely to be most beneficial when applied to patients with minimal levels of disease and tumor types known to be particularly immunogenic, such as melanoma and RCC. Safety issues must be evaluated in patients where no conventional treatment is proved to be successful; however, as we move from the realm of pilot studies, it will be critical to design future trials to tackle the subject of residual disease burden, which may occur after surgery. Preliminary research suggests that these therapies will be less toxic than more conventional modes of treatment.

Currently the scope of cancer immunization is limited because most of the vaccines have targeted antigens that are restricted to a subset of patients. This fits in with the concept of personalized medicine. The proposal of designing a universal cancer vaccine to be effective against the entire cancer spectrum involves looking for an ideal tumor antigen that is expressed in most cancer cells, is restricted to cancer, and is crucial for cancer cell survival. This goal appears to be unrealistic, and even if feasible, it may not improve the management of cancer. Developments that may improve immune therapy of cancer are:

- Clinical trials of tumor cell vaccines in patients with minimal residual disease at high risk of relapse
- Translation of cellular approaches into reproducible clinical benefit
- More precise assays for the clinical and immunological response to cellular treatment
- Definitions of the most potent combinations of effector cells and cytokine enhancers

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Chapter 11
Gene Therapy of Cancer

11.1 Introduction

Gene therapy is defined as the transfer of defined genetic material to specific target cells of a patient for the ultimate purpose of preventing or altering a particular disease state (Jain 1998). It has three components: (1) identification of the gene that is mutated in a disease and obtaining a healthy copy of that gene, (2) carrier or delivery vehicle called vectors to deliver the healthy gene to a patient's cells, and (3) additional DNA elements that turn on the healthy gene in the right cells and at the right levels. Gene therapy usually involves in situ production of therapeutic proteins but some approaches require suppression of gene expression to achieve therapeutic effects. Applications of gene therapy would be narrow if confined only to transfer of defined genetic material to specific target cells using vectors, which are usually viral but several nonviral vectors are used as well. Genes and DNA can be introduced without the use of vectors, and various techniques are being used to modify the function of genes in vivo without gene transfer, e.g., gene repair. Gene medicines may modify the effects of genes. If one includes cell therapy, particularly with the use of genetically modified cells, the scope of gene therapy becomes much broader. As a further extension, one can include genetically modified bacteria for delivery of therapeutic agents. Gene therapy can now be combined with antisense techniques and RNA interference (RNAi), further increasing the therapeutic scope. Details of gene therapy techniques are described in detail in a special report on this topic (Jain 2013). Cancer, the most important application of gene therapy currently, is the topic of this chapter.

11.2 Strategies for Cancer Gene Therapy

Cancer gene therapy is the transfer of genetic material to the cells of an individual with the goal of eradicating cancer cells, both in the primary tumor and metastases. Various strategies for cancer gene therapy are listed in Table 11.1.

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_11,
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Table 11.1 Strategies for cancer gene therapy

Direct gene delivery to the tumor
Injection into tumor
Electroporation
Gene therapy vectors
Viral vectors
Nonviral vectors
Hematopoietic gene transfer
Genetic modification of human hematopoietic stem cells and progenitor cells
Gene-based strategies for immunotherapy of cancer (immunogene therapy)
Cytokine gene therapy
Gene-modified cancer cell vaccines
Inhibition of immunosuppressive function
Nucleic acid (DNA/RNA)-based cancer vaccines
Monoclonal antibody gene transfer
Transfer and expression of ICAM-1 molecules on tumors
Delivery of toxic genes to tumor cells for eradication (molecular chemotherapy)
Gene-directed enzyme prodrug therapy
Insertion of suicide genes and bystander killing effect
Drug-resistance genes as adjuncts to high-dose chemotherapy
Correction of genetic defects in cancer cells (mutation compensation)
Insertion of tumor suppressor genes
Eliminating expression of oncogenes
Gene suppression by antisense approaches
Targeted gene therapy for cancer
Directing therapy against targets that are expressed only in cancer cells
Cell-specific targeting via a molecular conjugate vector
Targeted liposome-mediated gene therapy
Transcriptional targeting for cancer gene therapy
Targeted epidermal growth factor (EGF)-mediated DNA delivery
Targeting gene expression to hypoxic tumor cells
Targeting gene expression selectively by using the progression-elevated gene-3 promoter
Targeted gene expression controlled by MRI-guided focused ultrasound
Targeting of therapeutic agents to virus-associated cancers
Targeting tumors with genetically modified T cells
Targeted site-specific delivery of anticancer genes by nanoparticles
Targeting gene therapy to tumor vasculature (antiangiogenesis)
Oncolysis by genetically altered microorganisms
Bacteria (tumor targeted) as novel anticancer vectors
Oncolysis by genetically engineered viruses
In vivo gene therapy of malignant tumors with heat shock protein gene
Gene therapy combined with radiotherapy
Modulation of gene expression to enhance apoptotic death after radiation
Spatial and temporal control of gene therapy using ionizing radiation
Enhancement of radiation-induced cell death
Genetic manipulation to reduce hypoxia, which makes tumor cells radioresistant

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11.3 Direct Gene Delivery to the Tumor

11.3.1 Injection into Tumor

The vector can be administered into the tumor, around the tumor, or into a compartment containing the tumor mass. This has the advantages of high local vector concentration with improved tumor transduction and limits vector dissemination. The following represent some of the methods of direct tumor delivery of genes:

- Direct injection of naked DNA
- Particle-mediated transfer with a gene gun
- Direct injection of a plasmid DNA–liposome complex
- Injection of viral vectors encoding genes for cytotoxic prodrug-activating enzymes
- Injection of genetically modified viruses for selective oncolysis of the tumor

11.3.1.1 Particle-Mediated In Vivo Gene Therapy for Cancer

Skin is the most easily accessible organ for gene transfer in both animals and humans. Gene gun-mediated DNA immunization induced antigen-specific cellular and humoral immune responses that reduced tumor metastases in animal models. The gene gun has also transferred genes to internal rat organs, such as the kidneys, pancreas, and liver. The technique would allow cytokines and other biological response modifiers to be delivered to cancer patients directly.

11.3.1.2 Direct Injection of Adenoviral Vectors

Concern has arisen about the adverse effects of adenoviral (Ad) vectors administered systemically. Moreover, the protein product of IL-2 gene, used with Ad vectors, is also associated with systemic toxicity. To avoid this adverse effect, Ad vector incorporating the IL-2 gene can be injected directly into the tumor. Animal studies have shown improved efficacy and decreased toxicity of IL-2 in this treatment approach. Results of clinical trials have shown that no significant adverse effects are associated with the administration of the Ad IL-2 product in the recommended doses.

This procedure is limited to tumors that are accessible for direct injection such as those involving brain and the prostate. Animal studies have shown that direct injection of an Ad vector (Adv.RSV-tk) expressing the herpes thymidine kinase (tk) gene into established tumors in the liver, followed by systemic ganciclovir administration, is effective in inducing tumor necrosis. Toxicities are minimal at therapeutically effective vector doses, although severe hepatic necroinflammation is seen at much higher supratherapeutic doses.

A potential problem of intratumoral infusion is that viral vectors may disseminate from tumor to normal tissues during and after the infusion. To reduce the dissemination, a novel method has been developed based on a biocompatible polymer, poloxamer 407, which significantly increases the viscosity of virus suspension when the temperature is raised from 4 to 37 °C (Wang et al. 2005). This method significantly increases transgene expression in solid tumors and reduces virus dissemination by two orders of magnitude after intratumoral infusion of Ad vectors. The mechanism of reduction is likely to be that the viscous poloxamer solution blocks convection of viral vectors in the interstitial space and the lumen of microvessels in the vicinity of the infusion site. This method has a potential to be used clinically for enhancing efficacy and reducing toxicity of viral gene therapy for cancer.

11.3.1.3 Direct Injection of a Plasmid DNA–Liposome Complex

Direct injection of plasmid DNA–liposome complex into the tumor (in situ lipofection) has been reported in animal models and human clinical trials. Preinjection with cisplatin has been found to enhance the in situ lipofection of human ovarian carcinoma cells grown in SCID mice. Allovectin-7 (a plasmid DNA encoding the genes HLA-B7 and beta2-microglobulin complexed with a cationic lipid mixture, DMRIE/DOPE) has been used effectively and safely in metastatic melanoma in phase II clinical trials.

11.3.2 Electroporation for Cancer Gene Therapy

Electroporation involves applying pulsed electric fields across target cells to transiently increase the permeability of their surface membranes. This permeability facilitates the delivery of drugs or genes into the interior of the cells. In vivo electrogene therapy has been carried out with partial systemic immunity induced by direct injection of plasmid DNAs encoding mouse interleukin-2 (IL-2) and granulocyte–macrophage colony-stimulating factor (GM-CSF) in murine hepatoma models. The therapeutic genes delivered have also included cytotoxic genes, making possible a wide range of therapeutic strategies. Moreover, systemic antitumor effects were also observed, suggesting that this approach may be effective for the treatment of metastatic as well as primary tumors.

IL-12 is one of the most effective cytokines for treating malignancy. Intratumoral delivery of the murine IL-12 gene, using electroporation, has been found effective in inducing regression of established tumors in mice and more effective than intramuscular injection of this gene by electroporation. The gene expression profiles of tumors treated with intratumoral IL-12 electroporation gene therapy show that the three genes that are the most altered at the level of expression are Mig (Cxcl9), STAT1, and IRF7. Further studies indicate that these three genes may positively correlate with the antitumor efficacy of intratumoral IL-12 electroporation gene therapy.

Ichor Medical Systems Inc.'s patented TriGrid electrode array system is designed to improve the results achieved with electroporation by maximizing field uniformity within a targeted volume of tissue. As a result, the electroporation effect can be achieved throughout the diseased area with few side effects due to the electrical fields propagated within the tissue. By increasing the number of simultaneously active electrodes within a geometrically optimized array and sequencing their activation pattern, performance can be significantly enhanced, allowing electroporation therapy to be applied in a safe, efficient manner. A preclinical study was performed to assess the effectiveness of different electrode array systems. Fischer rats with a 9L gliosarcoma brain tumor were utilized to assess the effectiveness of electroporation in the delivery of the chemotherapeutic drug bleomycin to tumor tissue. It was concluded that the therapeutic effect of electroporation can be significantly improved using the electrical fields characteristic of the TriGrid array. In preclinical studies, Ichor scientists have demonstrated that direct delivery of the IL-12 gene into a local lesion can induce a significant reduction in the tumor mass. Not only did the local tumor respond, but the therapy also caused systemic anticancer effects, which prevented development of distant metastases. Importantly these effects were achieved without evidence of toxicity typically associated with IL-12 administration. The dramatic enhancement of immune responses compared to conventional injection, which has been extensively documented in animal models, has been confirmed in a randomized, placebo-controlled human clinical trial of an HIV DNA vaccine.

11.3.3 Control of Gene Expression in Tumor by Local Heat

Among the techniques used to induce and control gene expression, a noninvasive, physical approach based on local heat in combination with a heat-sensitive promoter represents a promising alternative but requires accurate temperature control in vivo. MRI-guided focused ultrasound (MRI-FUS) with real-time feedback control enables automatic execution of a predefined temperature-time trajectory. Temporal and spatial control of transgene expression, based on a well-defined local hyperthermia generated by MRI-FUS, has been demonstrated. Two cell lines were derived from C6 glioma cells.

11.3.4 Radiation-Guided Gene Therapy of Cancer

Radiation can be administered to selectively induce cytotoxic gene expression in the targeted tumor tissues. With promising results from phase II clinical trials using TNF-expressing Ad, it is possible to have radiation-guided gene therapy regimes once the tumor-targeted delivery has been achieved (Han et al. 2006). Tumor endothelium is an attractive biological target for gene therapy, because it has the advantage of stability, accessibility, and bioavailability for therapeutic agents. Technological advances in

DNA microarrays, proteomic profiling, and phage-displayed libraries have accelerated the identification of tumor-specific endothelial biomarkers and discovery of its relevant affinity reagents for targeted delivery. The application of radiation-guided gene delivery, its amplification, as well as expression of gene therapy presents great opportunities to be employed as an alternative cancer treatment.

11.3.5 Radioprotective Gene Therapy

Adverse effects of radiation such as myelosuppression or mucositis limit the effectiveness of radiotherapy by requiring dose reduction. Transfer of a radioprotective gene into normal tissue cells enables the reduction of risks associated with hematopoietic or intestinal toxicity after irradiation. Several potentially radioprotective genes such as multidrug resistance 1 (MDR1), snail homolog 2 (SNAI2), and superoxide dismutases have been evaluated in preclinical models for their radioprotective effect (Maier et al. 2011). Various viral vectors have been used for gene transfer. Further vector optimization for targeted cell-specific transduction and for more stable or regulated transgene expression is still needed. However, radioprotective gene therapy remains a promising method for reducing radiotherapy-related cytotoxicity of normal tissue cells and thus may improve success of anticancer therapy.

11.3.6 Nanoparticles to Facilitate Combination of Hyperthermia and Gene Therapy

Hyperthermia can be produced by near-infrared laser irradiation of gold nanoparticles present in tumors and thus induce tumor cell killing via a bystander effect. To be clinically relevant, however, several problems still need to be resolved. In particular, selective delivery and physical targeting of gold nanoparticles to tumor cells are necessary to improve therapeutic selectivity. Considerable progress has been made with respect to retargeting adenoviral vectors for cancer gene therapy. Covalent coupling of gold nanoparticles to retargeted Ad vectors enables selective delivery of the gold nanoparticles to tumor cells while retaining virus infectivity and ability to retarget tumor-associated antigens. This facilitates the use of hyperthermia and gene therapy as a combinatorial therapeutic approach.

11.4 Cell-Based Cancer Gene Therapy

Cell and gene therapies are intertwined for the treatment of cancer. Many gene therapies are based either on patients' cells or tumor cells. Immunogene therapy and cancer vaccines involve cells (see Chap. 7). Table 11.2 shows classification of cell-based gene therapy for cancer.

Table 11.2 Cell-based gene therapy for cancer

Genetically modified cells secreting anticancer agents
Genetically engineered cells secreting cytokines
Genetically modified encapsulated cells secreting enzymes for activating anticancer prodrugs
Cell-based cancer vaccines
Cytotoxic T cells: hybrid cell vaccination
Tumor-infiltrating lymphocytes
Genetically targeted T cells
NK (natural killer) cells
Dendritic cell (DC) vaccines
DCs pulsed with tumor lysate
DCs pulsed with apoptotic genes
Tumor cell vaccines
Tumor cells fused with DCs
Tumor cells transfected with cytokines
Genetically modified tumor cell vaccines
Stem cell-based anticancer gene therapies
Delivery of anticancer agents by genetically engineered mesenchymal stem cells
Hematopoietic progenitor cells with retroviral MDR 1 coexpression vectors

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11.4.1 Genetic Modification of Human Hematopoietic Stem Cells

The hematopoietic stem cell (HSC) is an ideal candidate for gene therapy. Transfer of genes is accomplished with retroviral or adenoviral vectors. In some studies, transferred genes are used as markers following a transplant to identify the source of stem cells (transplanted or endogenous) or the source of malignant cells.

Gene transfer into human HSCs enables enhancement of anticancer immunity, whereas alteration of HSCs may increase their resistance to cytotoxic drugs. MDR genes can be introduced into HSCs to reduce chemotoxicity and enable the administration of higher doses of chemotherapy. Tumor cell eradication can also be enhanced by genetic modification of chemosensitivity and immunomodulation. HSCs, genetically engineered with tumor-specific receptors, have been transplanted into mice injected with malignant tumor cells. HSCs produce a variety of immune cells that selectively target and destroy the cancer. This work has been done at Cell Genesys. Plans are under way for a clinical trial in cancer patients.

Excessive proliferative demands placed on HSCs by repeated cycles of chemotherapy or hematopoietic transplantation will increase HSC doubling and, under extreme conditions, can lead to HSC proliferative senescence. The following strategies have been developed to prevent HSC damage:

- Protection of HSCs from radiation and cyclophosphamide in animal models
- Introduction of MDR genes into HSCs by retroviral vectors
- Autologous transplantation with bone marrow

Hematopoietic cells differentiate in steps marked by the acquisition or loss of specific phenotypic characteristics. It is possible to efficiently purify cells with characteristics of HSCs by using techniques combining cytokine stimulation with antimetabolic treatments. Cytokines have also been shown to improve gene transfer to human HSCs.

In recent years HSCs have been the object of new research efforts and scientific advances. Therapeutic strategies have been set up using HSCs for the treatment of solid tumors such as ovarian cancer. In this context different approaches have been proposed and clinically investigated. The "autologous" approach refers to the use of HSCs as hematological support to high-dose chemotherapy regimens and to the use of HSCs as an abundant source of dendritic cells (DCs) for cancer vaccination protocols. Examples include high-dose chemotherapy with autologous HSC transplantation as first-line treatment of advanced ovarian cancer and in the use of cytokines both for the HSC collection and for the posttransplantation hematopoietic recovery.

11.5 Immunogene Therapy

The immune system is involved in the normal surveillance and suppression of cancer. Cancer cells can be modulated in such a way that they stimulate the cells of the immune system to recognize and destroy the malignant cells. One of the major goals of cancer immunotherapy is the induction of tumor-specific T lymphocyte responses that will be effective in the rejection of established tumors. The prospects for this therapy rely on the identification of tumor antigens.

Immunogene therapy is the most pursued form of cancer gene therapy in current clinical trials. Most of the genetic approaches to cancer immunotherapy are ex vivo therapies involving the use of tumor cells transduced with genes for immunostimulatory molecules using viral vectors. After irradiation, gene-modified tumor cells are reimplanted. Immunogene therapy includes the following:

- Cytokine gene therapy
- MAb gene transfer
- Transfer and expression of ICAM-1 (intercellular adhesion molecule-1) molecules on tumors
- Antisense inhibition of immunosuppressive function

In vivo immunogene therapy may involve either of the following strategies:

- Direct injection of cytokine gene vectors into tumors
- Naked DNA injection
- Nucleic acid-based cancer vaccines

11.5.1 Cytokine Gene Therapy

Cytokines are hormones that act primarily within the immune system. They are produced mainly by lymphocytes (lymphokines) and monocytes (monokines).

Cytokines can modulate the intensity of the immune response. Cytokines and related immunomodulators have been shown to produce antitumor effects in various types of cancers. The reasons for this ability are as follows:

- They directly inhibit tumor growth, e.g., IFN-α.
- They deliver immune factors to tumor site, e.g., IL-2.
- They augment the effector function of T cells in recognizing MHC-presented peptides on tumor cells.

Immunotherapy with cytokine-secreting tumor vaccines is considered to be most beneficial in patients whose tumors have been resected and in whom immune responsiveness has been restored. This treatment would serve as an adjuvant therapy to prevent the growth of micrometastases rather than cause the regression of established tumors. The role of cytokines in gene therapy can be summarized as follows:

- Retroviral vectors can be used to introduce cytokine genes into human tumor cells.
- Transduced tumor cells constitutionally express cytokines for several months.
- Cytokine-secreting tumor cells can be irradiated and will continue to secrete cytokines for several months.
- Rejection of cytokine-secreting tumor cells by nude mice and upregulation of major histocompatibility antigens on tumor cells demonstrate that the secreted cytokines are biologically active.

The following approaches have been used for immunomodulation of tumors through cytokine-mediated gene therapy.

IL-2. This is an important example of cytokine gene therapy. One major drawback of IL-2 is severe toxicity associated with systemic administration of IL-2. To circumvent this problem, multiple intratumor injections have been used, but these injections are impractical, particularly when tumor sites are not easily accessible. To avoid these difficulties, gene transfer has been explored as an alternative method of cytokine delivery.

One of the most intensively studied approaches to cytokine gene transfer into tumor cells is the use of the IL-2 gene. IL-2 production by tumor cells may confer immunogenicity, and such cells can be used as vaccines in cancer patients. Use of a recombinant vaccinia virus expressing human IL-2 has been shown to protect mice against implantation with neuroblastoma cells and may be useful for cancer gene therapy. Transduction can be efficiently accomplished by an adenoviral vector containing the human IL-2 gene, and high IL-2 expression was observed in freshly isolated human lung adenocarcinoma cells. This finding indicates a potentially useful but limited clinical role for this approach in gene therapy for patients with lung cancer. The IL-2 gene has also been successfully delivered into established human tumor xenografts in SCID mice by cationic liposome-mediated delivery. Preclinical studies demonstrate that intratumoral delivery of adenovirus expressing IL-2 eradicates preestablished tumors in mice and confers immune protection from rechallenge.

IL-7. This appears to be a cofactor promoting B-cell immunoglobulin rearrangement. Both immature and mature T cells also respond to IL-7. IL-7 has properties that would make it useful for immunotherapy of cancer, particularly in the gene therapy of cancer. These properties can be summarized as follows:

- Promotes strong proliferative and cytolytic antigen-specific T-cell response
- Generates moderate lymphokine-activated killer (LAK) cell toxicity with little effect on natural killer (NK) cells
- Accelerates recovery of lymphoid and myeloid systems after iatrogenic depletion

IL-12. Formerly called NK cell stimulator factor, IL-12 is produced primarily by stimulated macrophages. Emerging experimental evidence suggests that the cytokine IL-12 can enhance the development of an effective immune response against tumors. IL-12 exerts a potent antitumor effect following local or systemic administration. The major adverse effects are related to capillary leak syndrome, a side effect that limits its usefulness. Gene gun-mediated delivery of plasmid encoding IL-12 to intradermal tumors in mice has shown that gene therapy provides a safer alternative to IL-12 protein therapy for clinical treatment of cancers.

Microencapsulated murine fibroblast cells have been genetically engineered to produce IL-12 and act as a source of continuous release of IL-12. These cells have a significant therapeutic effect on the experimental colon tumor by activating antitumor immune responses in vivo. Microencapsulated and genetically engineered cells secreting cytokines may thus be an extremely versatile tool for tumor gene therapy.

Adeno-associated virus (AAV) vectors have been shown to be effective vehicles for delivering the IL-12 gene to leukemic cells, and these cells can be used to generate cancer vaccines. Delivery by retroviral vectors allows high-level expression and effective eradication of established tumors in multiple murine tumor models. Initial results of clinical trials studying Ad-mediated in vivo gene transfer of IL-12 into lesions of advanced cancer patients are encouraging. Further improvements are expected to result from (1) increases in the efficacy of gene transduction, (2) development of tumor-specific promoters, (3) development of regulatable long-term expression vectors, and (4) combination with other anticancer therapies.

IL-12. IL-12 is known to have an essential role in inflammatory responses and innate resistance to infection and cancer, which has been largely attributed to its ability to initiate the differentiation of T helper-1 cells producing IFN-γ. Several therapeutic strategies have been developed using IL-12 family members in cancer with special focus on gene administration (Waldner and Neurath 2009).

IL-13. The effects of this human T lymphocyte-derived cytokine in vivo have yet to be described. IL-13 has also been classified as a lymphokine. When delivered locally at the tumor site in a plasmid expression vector, it has shown striking antitumoral activity in a number of mouse models. The exact mechanism of action is not known, but IL-13 stimulates the sensitization of specific memory T lymphocytes that are responsible for long-lasting antitumor protection. Indirect action of IL-13 may occur via upregulation of other cytokines such as IL-12.

IL-21. This is a key factor in the transition between innate and adaptive immune responses. IL-21 is now developed as a cancer immunotherapeutic drug, but conditions for efficacious therapy, and the conflicting immunostimulatory and immunoinhibitory influence of the cytokine, are yet to be defined. IL-21 immunotherapy warrants clinical evaluation as a potential treatment for cancer.

IL-23. This is a novel cytokine composed of a newly identified p19 molecule and the p40 subunit of IL-12 that can stimulate the proliferation in vitro of memory T cells. Expression of IL-23 in tumors produces T cell-dependent antitumor effects and induces systemic immunity.

mda-7/IL-24. This molecule belongs to the IL-10-related family of cytokines. Extensive in vitro and in vivo human tumor xenograft studies have established its transformed cell apoptosis-inducing capacity in various model systems. It has a considerable cancer gene therapeutic potential and is in phase I/II clinical trials.

Combination of cytokines. Significant effort has focused on evaluating the use of exogenous cytokines, administered either systemically or locally into the tumor site via gene therapy. Several cytokines have demonstrated unique activity in the preclinical setting, using IFN-α-inducing cytokines such as IL-12 and IL-18. Most notably, later studies have now attempted to build on the clinical efficacy of IL-2 alone, to define combinations of agents with synergistic immunoregulatory and/or antitumor efficacy. Several lines of evidence suggest that IL-12 and IL-2 provide complementary immunoregulatory signals and have now shown that in combination, these two cytokines mediate synergistic antitumor activity in preclinical tumor models.

IFN-γ gene transfer. IFN-γ is a lymphokine secreted by T lymphocytes that serves as an activation and stimulation factor for many immune cells that may be cytotoxic for tumor cells. Murine γ-IFN gene transfer into murine tumor cell lines has been shown to trigger an immune response and boost the tumor-specific immunity in vivo. Similarly, delivery of the human γ-IFN gene to human melanoma cells will stimulate the cytolytic activity of CTLs from melanoma patients in vitro. This approach is being tested in clinical trials.

A retroviral vector containing a human γ-IFN gene has been used to transduce renal carcinoma cell lines; this approach was associated with increased production of γ-IFN and expression of human leukocyte antigen (HLA) class I and class II molecules. These effects are retained after irradiation and cryopreservation. The results suggest that an autologous tumor cell vaccine trial with irradiated γ-IFN gene-transduced renal carcinoma cells is rational and feasible.

Use of TGF-β insensitive immune cells in gene therapy. TGF-β is a multifunctional cytokine. At a cellular level, it mediates cellular proliferation, growth arrest, differentiation, and apoptosis. Because of the above cellular effects, TGF-β is able to regulate a host of pathophysiological events in vivo, such as normal embryonic development, angiogenesis in tumor tissues, malignant transformation, and immune surveillance. As a general rule, its direct effect on cancer cells is inhibition to cancer growth. However, cancer cells are able to acquire the ability to evade this inhibitory effect of TGF-β by becoming insensitive to TGF-β. Furthermore, these malignant

cells are able to produce large quantities of TGF-β. The consequence of overexpression of TGF-β by cancer cells is an important factor for subsequent tumor progression. The excess amount of TGF-β promotes tumor angiogenesis and immune suppression. The latter effect of TGF-β is the most devastating to the host. The host immune system offers a natural defense program against cancer. But this natural immune surveillance is rendered ineffective by an overproduction of TGF-β derived from the tumor cells. Rendering the host immune cells insensitive to TGF-β in a gene therapy program offers a hope to successfully combat against cancer.

Granulocyte–macrophage colony-stimulating factor. GM-CSF is a potent naturally occurring protein that is most often associated with the growth and differentiation of hematopoietic progenitors. GM-CSF helps orchestrate immune responses and has been shown in preclinical and clinical studies to boost the immune system's ability to recognize and destroy tumor cells. Irradiated melanoma cells infected with recombinant vaccinia virus expressing GM-CSF can inhibit small metastatic tumors in mice. Particle-mediated transfection of fresh tumor explants with GM-CSF cDNA is an effective and clinically attractive approach for cancer gene therapy. Partial systemic immunity can be provoked by IL-2/GM-CSF double gene electrogene therapy. This has the potential for future clinical applications.

11.5.2 Genetically Modified Cancer Cell Vaccines

A more recent approach is the use of vaccines containing genetically modified cells. The patient's immune system should distinguish between healthy and tumor cells and recognizes the tumor cells as foreign or diseased. However, sometimes these tumor cells are able to induce tolerance and to escape recognition and destruction by the innate and acquired immune system. The aim of many cell/gene therapy approaches is to design therapeutic vaccines that enable the immune system to recognize and destroy tumor cells, just as it can destroy cells infected with common viral diseases. To remove the tolerance of tumor cells by the patient's immune system, these tumor cells need to be modified by the transfer of genes. These genetically modified tumor cells are then injected into the patients. The most common gene-modified vaccines use cytokines—the cytokine is produced in high concentrations in the vicinity of the tumor cells, where it alters the local immunological environment and enhances the activities of antigen-presenting cells and the activation of tumor-specific T cells. This approach avoids the side effects associated with systemic treatment with cytokines.

11.5.3 Genetically Modified Dendritic Cell Vaccines

Gene therapy techniques can be applied to DC vaccines; such techniques use recombinant viral vectors that are incapable of replication to provide efficient and reliable

means of gene transfer. Genetic material is introduced into DCs to provide them with a renewable source of antigen for presentation; this should lead to more sustained expression of antigen. The expression of viral (and therefore foreign) genes may boost the immune response, but this antiviral immunity primed by DCs may cause the immune system to destroy DCs rapidly in subsequent rounds of immunization. One solution may be to use viral vectors that do not result in the expression of viral genes, such as retroviruses or "gutless" adenoviral vectors.

Lentivirus vectors can be used for genetic modification of human DCs and have an advantage over retroviral vectors in that they do not require target replication for efficient transduction. Using lentiviral vectors, efficient gene transfer in DCs can be obtained, and that these DCs can elicit antigen-specific immune responses in vitro and in vivo. The composition of the transfer vector has a major impact on the transduction efficiency.

AAVs can be used to transduce human DCs, and their main advantage is a decrease in viral-derived epitopes leading to decreased immunogenicity of the vector. However, DCs transduced with an adenovirus expressing a tumor antigen can completely protect the host from tumor challenge. This method also avoids the Ad-associated hepatic toxicity caused by direct administration of Ad vectors. Vector-mediated in vivo activation and tumor-associated antigen loading of DCs do not require additional cytokine boosting to induce the immune response against the tumor cells. This vector strategy may therefore be of use in the development of immunotherapy for the many carcinomas in which the hMUC-1 antigen is overexpressed.

11.5.4 Nucleic Acid-Based Cancer Vaccines

Use of therapeutic vaccines based on nucleic acids (DNA or RNA) is considered to be genetic immunization or gene therapy. There are three major types of synthetic and recombinant cancer vaccines: recombinant viral and bacterial vaccines, naked DNA or RNA vaccines, and recombinant protein and peptide vaccines.

11.5.4.1 DNA Cancer Vaccines

Delivery of antigens by injection of the encoding DNA allows access to multiple antigen-presenting pathways. Knowledge of immunological processes can therefore be used to modify construct design to induce selected effector functions. Expression can be directed to specific intracellular sites, and additional genes can be fused or co-delivered to amplify responses. Therapeutic vaccination against cancer adds a requirement to overcome tolerance and to activate a weakened immune repertoire. To activate immunity against tumor antigens, tumor-derived sequences can be fused to genes encoding microbial proteins. This strategy engages T helper cells from the large antimicrobial repertoire for linked help for inducing antibody against cell surface tumor antigens. Epitope-specific DNA vaccination leads to powerful antitumor

attack. Vaccine designs validated in preclinical models are now in clinical trials with immune responses detected against both tumor antigens and fused microbial antigens. DNA priming is highly efficient, but boosting may benefit from increased antigen expression. Physical methods including electroporation provide increased expression without introducing additional competing antigens. A wide range of cancers can be targeted, and objective assays of response will determine efficacy.

11.5.4.2 RNA Vaccines

The use of RNA has been proposed for use in tumor vaccination protocols. The use of RNA has several potential advantages. Since total cellular RNA or mRNA can be utilized, it is not necessary to know the molecular nature of the putative tumor antigen(s). RNA can be effectively amplified; thus, unlike tumor-extract vaccines, only a small amount of tumor is needed to prepare the material for vaccination. Also, unlike DNA-based vaccines, there is little danger of incorporation of RNA sequences into the host genome. The possible utility of RNA-based vaccines for tumor immunotherapy should be further explored to determine whether such approaches are clinically useful. RNA can be used in the form of antigen with which to load DCs for cancer vaccines.

11.5.5 Viral Vector-Based Cancer Vaccines

Transgene uses viral vectors for cancer vaccines, which are now in clinical trials. MVA vaccinia virus vector is a highly attenuated poxvirus that combines the advantages of a strain extensively tested in humans as a smallpox (vaccinia) vaccine with the ability to stimulate a strong immune response to antigens. The sequence coding for the cytokine IL-2 is included to help stimulate specific T-cell responses. The MVA-Muc1-IL2 vaccine candidate uses the MVA vector to express the Muc1 tumor-associated antigen found in most adenocarcinomas. The purpose of treating cancer patients with MVA-Muc1-IL2 is to induce MUC1 antigen expression in a nontumor environment, i.e., where the immune system is fully functional, in order to induce both innate and adaptive immunity. The MVA-Muc1-IL2 cancer vaccine (TG4010) is in clinical trials for the treatment of metastatic NSCLC in combination with first-line chemotherapy.

The MVA-HPV-IL2 vaccine candidate uses the MVA vector to express the E6 and E7 antigens of the human papilloma virus 16, an agent causally linked to cancer and precancerous lesions of the cervix. Monotherapy with MVA-HPV-IL2 is being evaluated in three phase II clinical trials in patients with high-grade cervical dysplasia according to different doses and treatment modalities.

11.5.5.1 Intradermal Delivery of Cancer Vaccines by Ad Vectors

The recombinant Ad vector is being considered as a cancer vaccine platform because it efficiently induces immune responses to tumor antigens by intradermal immunization. A study has evaluated the potential toxicities and biodistribution after a single dose or 6 weekly intradermal doses of Ad2/gp100v2 and Ad2/MART-1v2, which encode tumor-associated antigens gp100 and MelanA/MART-1, respectively (Plog et al. 2006). The only dose-related toxicities associated with intradermal administration of these Ad vectors were inflammatory cell infiltrates in the draining lymph nodes and injection sites that persisted for a few months after administration. The biodistribution of Ad DNA as detected by real-time PCR was largely confined to the injection sites and draining lymph nodes of mice treated with either a single dose or multiple doses of Ad vector and in the spleens of mice treated with multiple doses of Ad vector. Ad DNA was transiently detected in the bone marrow, lung, or blood of only one animal for each site and was below the limit of assay quantification. The vector persisted in the skin and lymph nodes as long as 3 months after the last dose. It is concluded that Ad vectors delivered by intradermal administration provide a safe, genetic vaccine delivery platform that induces desirable immune responses at the immunization sites and the lymph nodes that, ultimately, result in immune responses specific to the tumor antigens. This technique is in development at Genzyme Corporation.

11.6 Monoclonal Antibody Gene Transfer for Cancer

The presence of immune response to cancer is well recognized, but it is usually weak because it fails to elicit T-cell helper response. This response involves the release of necessary cytokines to stimulate the production of cytolytic T cells that can destroy tumors. Tumor cells are capable of producing self-reactive antibodies following MAb gene transfer. Several toxic antibodies with different functional properties are promising candidates for MAb gene modification of tumor cells. Antibodies directed against T-cell activation antigens are well known for their stimulator properties in vitro and in vivo. In preparation of a clinical phase I/II study in renal cell carcinoma patients, a clinically applicable protocol was developed for the expansion of primary human T lymphocytes.

Antibody-mediated targeting has been attempted by use of replication-competent retroviral vectors. Murine leukemia virus (MLV) vectors can be engineered to achieve high-efficiency gene transfer to solid tumors in vivo. However, application of antibody-mediated targeting to the initial localization of replication-competent virus vectors to tumor sites requires optimized target selection and vector design.

11.7 Other Gene-Based Techniques of Immunotherapy of Cancer

Other techniques of immunotherapy of cancer relevant to gene therapy are described here briefly. Some of these techniques may be combined with immunogene therapy.

11.7.1 Fas (Apo-1)

Fas, a member of the TNF receptor/nerve growth factor receptor superfamily, signals apoptosis in susceptible target cells when bound by Fas ligand (FasL) or agonistic antibodies. Preexposure of the glioma cell lines to cytokines IFN and TNF, which sensitize for Fas-dependent killing, has been shown to partially overcome bcl-2-mediated rescue from apoptosis. While Fas ligand gene transfer indeed eliminates cancer cells and inflammatory cells through apoptosis, it also kills normal cells and initiates inflammation in certain tissues. Thus, new strategies that can modify the apoptotic or proinflammatory activities of the FasL will help to fully realize the potential of the FasL gene therapy.

11.7.2 Chemokines

Chemokines are a superfamily of small proteins secreted mainly by leukocytes. Chemokine gene transfection into tumor cells has been shown to reduce tumorigenicity in nude mice in association with neutrophilic infiltration. Chemokines may be useful in combination with anticancer agents or other types of cytokines, such as IL-2, IFNs, and colony-stimulating factors, because they have a different antitumor mechanism and are well tolerated at high doses. Antigenic stimulation via the major histocompatibility complex class I molecules, cytokine, and chemokine combination may provide a new and promising approach to cancer gene therapy, which is more likely to bypass tumor immunosuppression mechanisms.

Genetically modified lymphocytes are a new class of killer cells. Lymphocytes can be genetically modified to produce and secrete a targeted toxin against tumor cells. The transduced lymphocytes have a potent and selective cytotoxic effect on tumors in culture and nude mouse models, probably because of the production and accumulation of targeted toxin inside the tumors. This approach, which has the features of both an antibody-directed and cell-mediated immunotherapy, may have applications in cancer gene therapy. The administration of highly avid antitumor autologous lymphocyte populations can be far more active in mediating tumor regression in vivo when administered after nonmyeloablative chemotherapy than when administered without this prior chemotherapy, and intra-arterial administration

of autologous lymphocytes into the blood supply of the tumor can be more effective in mediating tumor regression than the intravenous administration of these same tumor-infiltrating lymphocytes. Gene-targeted primary T lymphocytes depict specific functional activity against autologous colorectal tumor cells. Chimeric immune receptor-expressing T cells may be able to circumvent the mechanisms used by tumor cells to avoid immune cell activity in vivo.

11.8 Inhibition of Immunosuppressive Function in Cancer

TGF-β, which is expressed by a majority of malignant tumors, is the most potent immunosuppressor and, therefore, the most likely cytokine to be responsible for the latter phenomenon. In addition to playing a key role in tumor-induced immunosuppression, TGF-β stimulates angiogenesis. However, tumor cells eventually become refractory to TGF-β-mediated growth arrest, either due to loss of TGF-β receptors or due to dysregulation in TGF-β signaling pathways. Neutralization of TGF-β or inhibition of its production is an effective method of cancer treatment in a variety of animal models. Several agents targeting TGF-β are in the early stages of development and include anti-TGF-β antibodies, small molecule inhibitors of TGF-β, Smad inhibitors, and antisense gene therapy. Since tumors may express more than one isoform of TGF-β, these new drugs should target all three TGF-β isoforms produced by human tumors. The effects of therapies targeting TGF-β are likely to be synergistic with cytotoxic chemotherapy and immunotherapy. Reversal of TGF-β-induced immunosuppression is a promising approach to cancer therapy.

11.9 Delivery of Toxic Genes to Tumor Cells for Eradication

11.9.1 Gene-Directed Enzyme Prodrug Therapy

Gene-directed enzyme prodrug therapy (GDEPT) only requires a fraction of the target cells to be genetically modified, providing that the resultant cytotoxic prodrug metabolites redistribute efficiently (the bystander effect). This transfer of cytotoxicity to neighboring nontargeted cancer cells is central to the success of any gene therapy strategy, irrespective of the therapeutic gene employed. Examples of prodrugs of clinically established chemotherapeutic agents currently used in conjunction with radiotherapy include 5-fluorocytosine, cyclophosphamide, irinotecan, gemcitabine, capecitabine, and mitomycin C.

Several combinations of enzyme/prodrug have been developed to improve the efficacy of molecular chemotherapy and overcome the limitations of tk/GCV as shown in Table 11.3. For example, some of the enzyme/prodrug combinations induce toxic effects not only in cycling but also in non-cycling cells (nitroreductase,

Table 11.3 Enzyme/prodrug combinations employed in suicide gene therapy

Enzyme	Prodrug	Drug	Mode of action
Carboxylesterase	CPT-11	SN38	Topoisomerase I inhibitor
Carboxypeptidase G2	Aromatic N-substituted glutamates	Benzoic acid, phenol, and aniline mustards	DNA cross-linking
Cytosine deaminase	5-Fluorocytosine	5-Fluorouracil	Blocks DNA/RNA synthesis (pyrimidine antagonist)
Cytochrome P450	Cyclophosphamide	Phosphoramide mustard	Blocks DNA synthesis (DNA alkylating agent)
HSV-thymidine kinase	Ganciclovir	Ganciclovir triphosphate	Blocks DNA synthesis
Nitroreductase	Nitrobenzyl-oxycarbonyl-anthracyclines (CB1954)	Anthracyclines	DNA cross-linking
Purine nucleoside phosphorylase	6-Mercaptopurine-DR	6-Mercaptopurine	Blocks DNA synthesis (purine antagonist)
Human β-glucuronidase	HMR 1826 (inactive derivative of doxorubicin)	Doxorubicin	Topoisomerase II inhibitor

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purine nucleoside phosphorylase). With others, the bystander effect (suicide gene therapy) is stronger (purine nucleoside phosphorylase) or does not require cell-to-cell contact (cytosine deaminase, nitroreductase, secreted form of lysosomal human β-glucuronidase).

11.10 Combination of Gene Therapy with Radiotherapy

In this strategy, designated genetic radiotherapy, radiation is combined with gene therapy to spatially and temporally control transgene expression in the irradiated field. Genetic radiotherapy is used for:

- Modulation of gene expression to enhance apoptotic death after radiation
- Spatial and temporal control of gene therapy using ionizing radiation
- Enhancement of radiation-induced cell death
- Genetic manipulation to reduce hypoxia (which makes tumor cells radioresistant)

11.11 Correction of Genetic Defects in Cancer Cells

Malignant transformation results from a series of accumulated, acquired genetic lesions, many of which have been identified. These genetic lesions fall into two general types: loss of expression of recessive tumor suppressor genes and aberrant

Table 11.4 Mutation compensation strategies used clinically

Target	Strategy	Vector	Tumor type
p53	Replacement of tumor suppressor gene	Adenovirus	Bladder cancer; breast cancer; glioma; hepatic metastases of colon cancer; hepatocellular carcinoma; non-small-cell lung cancer; ovarian cancer; prostate cancer; squamous cell carcinoma of the head and neck
p16	Replacement of tumor suppressor gene	Adenovirus	Prostate cancer
Rb (retinoblastoma) tumor	Replacement of tumor suppressor gene	Adenovirus	Bladder cancer
BRCA-1	Replacement of tumor suppressor gene	Retrovirus	Breast cancer; ovarian cancer; prostate cancer
Insulin-like growth factor 1	Antisense	Liposomes	Glioblastoma
K-ras	Antisense	Retrovirus	Non-small-cell lung cancer
Epidermal growth factor receptor	Antisense	Liposomes	Squamous cell carcinoma of the head and neck
Bcr/abl	Antisense	Retrovirus	Chronic myelogenous leukemia
c-myc, *c-fos*	Antisense	Retrovirus	Breast cancer
erbB-2	Intracellular single-chain antibody	Adenovirus	Ovarian cancer

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expression of dominant oncogenes. It is therefore rational to intervene at the genetic level to rectify these molecular lesions in the cancer cell, a strategy broadly defined as mutation compensation. Various mutation compensation strategies used clinically are shown in Table 11.4.

11.12 Targeted Gene Therapy for Cancer

Several technologies have been tried for targeted gene therapy. A few of these will be described briefly.

11.12.1 Antiangiogenic Therapy for Cancer

A growing tumor needs an increased blood supply for its proliferating cells. Tumor vessels possess unique physiological features that might be exploited for improved therapeutic delivery. Inhibition of angiogenesis seems to be a relatively safe therapeutic option against cancers and has therefore become a logical arena for a wide

range of experimentation. MAbs and small molecular drugs are the most widely applied methods for inhibition of angiogenesis. The efficacy of these antiangiogenic modalities has been proven in both preclinical and clinical settings. Anti-VEGF MAb bevacizumab as a first-line treatment for metastatic colorectal cancer was approved more than a decade ago. Limitations of antiangiogenic MAb therapy include the following:

- Production of antiangiogenic MAbs is costly and difficult.
- Because of limited serum half-life, MAbs need to be administered at constant high doses over a prolonged period of time as repeated intravenous infusions with consequent adverse effects.
- Current antiangiogenic agents are intrinsically selective in their action on the VEGF pathway but are not specific.
- Resistance can develop to current antiangiogenic therapies by upregulation of alternative proangiogenic pathways.
- Rebound host response to antiangiogenic therapy might make some tumor cells more aggressive, invasive, and metastatic.

Gene therapy, applied in the antiangiogenic setting, can overcome some of the problems mentioned above (Samaranayake et al. 2010). Advantages of gene therapy are:

- Production of therapeutic agent at high concentration over a desired period of time at the local site, thereby reducing unwanted systemic adverse effects.
- Local administration of antiangiogenic gene therapy has the advantage of overcoming natural barriers for conventional drugs, such as the BBB in case of brain tumors.
- Systemic gene therapy can be targeted to the tumor using both viral and nonviral vectors.
- Low toxicity of gene therapy products makes them good candidates for combination with cytotoxic chemotherapies for enhancing therapeutic effect.
- As a one-time application, gene therapy could be more economical when compared with other pharmaceuticals.

Gene therapy, however, is not without adverse effects. There is the possibility of systemic toxicity with certain antiangiogenic gene therapies. As with other antiangiogenic agents, antiangiogenic gene therapies will most probably need to be combined with conventional chemotherapy and radiotherapy or with other gene therapy strategies that have synergistic effects. For example, adenovirus-mediated HSV-tk GCV suicide gene therapy has been combined successfully with adenovirus-mediated endostatin gene therapy. Implantation of genetically modified cells that secrete antiangiogenic agents would be another way to deliver antiangiogenic therapy. Cell encapsulation systems can be used to protect these cells from the immune system and regulatable systems can be incorporated to control gene expression in these systems (Samaranayake et al. 2009).

11.12.2 Bacteria as Novel Anticancer Gene Vectors

Most of the emphasis in gene therapy has been on viral and nonviral vectors such as liposomes. Investigation of bacteria as gene delivery vectors has been scant. Salmonella bacteria offer several potential advantages as anticancer vectors:

- Multiple tumors can be targeted from a distant inoculation site.
- Salmonella can grow under aerobic as well as anaerobic conditions such as those found in tumors.
- Salmonella has the ability to express suicide genes such as HSV-tk.

Wild-type Salmonella cannot be used for therapeutic purposes. When injected into mice bearing melanoma cells, the bacteria were found at high concentrations in the tumor but caused death of the animals. Genetically engineered Salmonella offer an intriguing new approach to selectively target solid tumors, including melanoma, lung, colon, breast, kidney, and liver. These bacteria target tumors after systemic administration and selectively replicate within them. Specificity for tumors is often more than 1,000 times greater than for any other tissue. An altered lipid greatly reduces the potential for septic shock yet also retains the antitumor properties of these bacteria. These bacteria have innate antitumor activity toward both primary and metastatic tumors and the ability to deliver proteins capable of activating chemotherapeutic agents directly within tumors. The delay in tumor growth results in mice that survive up to twice as long. These bacteria are susceptible to a wide range of antibiotics, allowing external control of the vector after administration.

11.12.3 Cancer-Specific Gene Expression

One impediment to effective cancer-specific gene therapy is the rarity of regulatory sequences targeting gene expression selectively in tumor cells. Although many tissue-specific promoters are recognized, few cancer-selective gene promoters are available. Progression-elevated gene-3 (PEG-3) is a rodent gene that displays elevated expression as a result of action of oncogenes, DNA damage, and cancer cell progression. The promoter of PEG-3, PEG-Prom, displays robust expression in a broad spectrum of human cancer cell lines with marginal expression in normal cellular counterparts. PEG-Prom-mediated expression of these genes kills only cancer cells and spares normal cells (Su et al. 2008). The efficacy of the PEG-Prom as part of a cancer gene therapeutic regimen is further documented by in vivo experiments in which PEG-Prom-controlled expression of an apoptosis-inducing gene completely inhibited prostate cancer xenograft growth in nude mice. These compelling observations indicate that the PEG-Prom, with its cancer-specific expression, provides a means of selectively delivering genes to cancer cells, thereby providing a crucial component in developing effective cancer gene therapies.

11.12.4 Cancer-Specific Transcription

Several strategies have been used to restrict transcription of transgenes to tumor cells. These include a range of promoters, which are tissue specific, tumor specific, or inducible by exogenous agents. Transcriptional targeting should prevent normal tissue toxicities associated with other cancer treatments, such as radiation and chemotherapy. In addition, the specificity of these strategies should provide improved targeting of metastatic tumors following systemic gene delivery. Rapid progress in the ability to specifically control transgenes will allow systemic gene delivery for cancer therapy to become a real possibility in the near future.

Cancer-specific promoters are useful tools to accomplish targeted expression; however, high levels of gene expression are needed to achieve therapeutic efficacy. Incorporating an imaging reporter gene in tandem with the therapeutic gene will allow tangible proof of principle that gene expression occurs at the correct location and at a sufficient level. Gene-based imaging can advance cancer detection and diagnosis. By combining the cancer-targeted imaging and therapeutic strategies, the exciting prospect of a "one-two punch" to find hidden, disseminated cancer cells and destroy them simultaneously can potentially be realized.

11.12.5 Delivery of Retroviral Particles Hitchhiking on T Cells

Antigen-specific T cells circulate freely and accumulate specifically at sites of antigen expression. To enhance the survival and targeting of systemically delivered viral vectors, a Mayo Clinic research team has exploited the observation that retroviral particles adhere nonspecifically, or "hitchhike," to the surface of T cells (Cole et al. 2005). By hitching a ride on the T cells, the therapeutic particles can hit their tumor target while avoiding detection (and destruction) by the immune system. Adoptive transfer of antigen-specific T cells, loaded with viruses encoding IL-12 or HSV-tk, cured established metastatic disease where adoptive T-cell transfer alone was not effective. Protection, concentration, and targeting of viruses by adsorption to cell carriers represent a new technique for systemic delivery of vectors, in fully immunocompetent hosts, for a variety of diseases in which delivery of genes may be therapeutically beneficial. This method opens up new opportunities for using viruses therapeutically because this method of attachment enables not only targeting of particular cells but also facilitating an entry into the cells, which is required to deliver therapeutic genes to destroy tumors. The T cells also help kill tumors.

11.12.6 Electrogene and Electrochemotherapy

One study has evaluated the feasibility and therapeutic potential of electrogene therapy with p53 alone or combined with electrochemotherapy using cisplatin on two

murine sarcomas with different p53 status (Grosel et al. 2006). Pretreatment of tumors with electrogene therapy with p53 enhanced chemosensitivity of both tumor models treated by electrochemotherapy with cisplatin. The combination of electrogene therapy and electrochemotherapy after only one application resulted in complete regression of tumors. Results of this study show that electrogene therapy with p53 alone or combined with electrochemotherapy is feasible and effective treatment of tumors.

11.12.7 Epidermal Growth Factor-Mediated DNA Delivery

The epidermal growth factor receptor (EGFR) is a rational target for cancer therapy because it is commonly expressed at a high level in a variety of solid tumors, and it has been implicated in the control of cell survival, proliferation, metastasis, and angiogenesis. However, despite evidence to suggest that EGFR expression is associated with a poor prognosis in some tumors (e.g., breast, head and neck carcinomas), the situation is by no means clear-cut. A number of issues are worthy of particular consideration, including how EGFR is measured and whether these assays are sensitive and reproducible, which mechanisms other than increased EGFR expression might cause the EGFR signaling drive to be increased, and the relationship, if any, between EGFR expression and the response to EGFR-targeted agents. However, tumors with EGFR expression are suitable for targeting with gene therapy. Targeted suicide gene therapy experiments with Ad vector in combination with ganciclovir (AdCMV-HSV-tk) enhances kill of osteosarcoma cell lines and primary short-term cultures severalfold by the EGFR-targeted vector.

11.12.8 Gene-Based Targeted Drug Delivery to Tumors

Ark Therapeutics Group's novel gene-based drug targeting platform technology Scavidin® has been shown to be highly effective in stopping tumor development in two cancer treatment models, using low doses of existing anticancer agents which would be subtherapeutic if administered conventionally. Scavidin® was used to target and concentrate intravenous doses of as little as one-tenth the conventional levels of the radioisotope Yttrium^{90} in one model, and the chemotherapy drug paclitaxel in another, to tumors growing under the skin.

Scavidin® is a novel two-part drug targeting technology originating from the DNA, which expresses the scavenger receptor on white blood cells. This natural receptor usually collects undesired fats and damaged cells and membranes from the blood, transporting them into the white blood cells and releasing them for destruction as part of the body's natural "cleanup" system. By modifying the DNA sequence for such receptor types, Ark has developed a new family of receptors which specifically bind only to the protein biotin, a naturally occurring substance which can easily be attached to therapeutic agents.

The Scavidin® DNA is put into the tumor where it expresses the new drug targeting receptor. The therapeutic agent, pretagged with biotin, is then given intravenously at low doses. As the therapeutic agent circulates round the body, Scavidin® extracts it from the blood by binding to the biotin tag, taking it into the cell and releasing it. The receptor then goes back and collects more. This revolutionary "molecular shuttle" system concentrates the therapeutic agent from a low and ineffective dose in the blood to a high therapeutic dose specifically in the target tissue. In this way, an important and highly effective therapeutic, which could have a poor safety profile (such as chemotherapy with high unwanted side effects) at a traditional dose, may be given in a low and safe dose systemically, with Scavidin® concentrating it specifically at the disease site where its treatment effect is needed. As such, it has enormous potential across many disease areas.

11.12.9 Gene Expression in Hypoxic Tumor Cells

Hypoxia activates a signaling cascade that culminates in the stabilization of the hypoxia-inducible factor (HIF)-1 transcription factor and activation of genes that possess a hypoxia response element (HRE). In GDEPT approach, bioreductive drugs that are preferentially toxic to tumor cells in a hypoxic environment are being evaluated in clinical trials; the lead compound, tirapazamine (TPZ), is being used in combination with cisplatin and/or with radiotherapy. Crucially, tumor response to TPZ is also dependent on the cellular complement of reductases. In particular, NADPH–cytochrome P450 reductase (P450R) plays a major role in the metabolic activation of TPZ. Using Ad delivery, human P450R has been overexpressed specifically within hypoxic cells in tumors, with the aim of harnessing hypoxia as a trigger for both enzyme expression and drug metabolism. The Ad used incorporates the hypoxia-responsive element (HRE) from the lactate dehydrogenase gene in a minimal SV40 promoter context upstream of the cDNA for P450R. In a human tumor model in which TPZ alone does not potentiate radiotherapeutic outcome, complete tumor regression has been observed when tumors were virally transduced before treatment.

11.12.10 Genetically Modified T Cells for Targeting Tumors

The genetic modification of T lymphocytes is an important approach to investigating normal T-cell biology and to increasing antitumor immunity. A number of genetic strategies aim to increase the recognition of tumor antigens, enhance antitumor activities, and prevent T-cell malfunction. T cells can also be engineered to increase safety, as well as to express markers that can be tracked by noninvasive imaging technologies. Genetically modified T cells are therefore proving to be of

great value for basic immunology and experimental immunotherapy. Cell-based carriers can be used to direct vector production to target sites for systemic therapy, e.g., T cells engineered to express a chimeric T-cell receptor can specifically recognize target cells expressing the tumor-associated CEA.

Human T cells targeted to the B-cell-specific CD19 antigen through retroviral-mediated transfer of a chimeric antigen receptor (CAR) called 19z1 have shown significant but partial in vivo antitumor efficacy in an acute lymphoblastic leukemia (ALL) model (Brentjens et al. 2007). The causes of treatment failure in this model were investigated, and approaches were designed to enhance the efficacy of this adoptive strategy. Expression of the 19-28z CAR, containing the signaling domain of the CD28 receptor, enhanced systemic T-cell antitumor activity when compared with 19z1 in treated mice. T-cell injections, designed to prolong in vivo T-cell function, further improved long-term survival. Thus combined in vivo costimulation and repeated administration enhance eradication of systemic tumor by genetically targeted T cells. The finding that modifications in CAR design as well as T-cell dosing enable the complete eradication of systemic disease affects the design of clinical trials using this treatment strategy. The investigators have an ongoing study using these T cells in CLL and are planning a trial in patients with ALL. The idea is that a patient's own T cells are taken and reeducated by inserting a gene into them that will enable them to produce a receptor to recognize B-cell cancers, and then they are returned to the patient where they should be able to attack and kill the tumor cells. Because the technique uses a patient's own T cells, there is little risk of compatibility issues or rejection, as there might be with human stem cell transplant. Human stem cell transplant, following radiation or chemotherapy, is currently incorporated into the treatment of several B-cell malignancies.

The extensive exploitation of the antitumor effect of donor lymphocytes infused after allogeneic HSC transplantation is limited by the risk of GVHD. To overcome this limitation, the therapeutic potential of donor lymphocytes engineered with the suicide gene tk of herpes simplex virus (HSV) was investigated in patients experiencing recurrence of hematological malignancies after allo-HSCT (Ciceri et al. 2007). The antitumor effect tightly correlated with the in vivo expansion of TK^{+} cells. Immunization against HSV-tk was observed in some patients but did not preclude an effective GvL. These data validate the feasibility, safety, and efficacy of TK(+) cells in the context of allografting and represent the basis for a broader application of this technology.

CARs combine the antigen binding site of a MAb with the signal activating machinery of a T cell, freeing antigen recognition from MHC restriction and thus breaking one of the barriers to more widespread application of cell therapy. T cells bearing CARs kill cells expressing target antigens on their surface, in a HLA-independent manner. T cells expressing CARs are highly targeted like MAbs but also offer the potential benefits of active trafficking to tumor sites, in vivo expansion, and long-term persistence. Furthermore, gene transfer allows the introduction of countermeasures to tumor immune evasion and of safety mechanisms (Ramos and Dotti 2011). CAR-modified T cells are likely play an increasing role in cell therapy of cancer.

11.12.11 Genetically Engineered Stem Cells for Targeting Tumors

Genetically engineered stem cells have been used to locate tumors and then produce biological killing agents right at the cancer site. Their novel treatment may offer the first gene therapy "delivery system" capable of homing in on and then attacking cancer that has metastasized wherever it is in a patient's body. The stem cells are not rejected, even if they are not derived from the patient.

The system has been tested in mice with a variety of human cancers including solid ones such as ovarian, brain, and breast cancer, melanoma, and even hematological cancers such as leukemia. These unspecialized cells can migrate to an injury by responding to signals from the area. There they develop the kind of connective tissue that is needed to repair the wound and can become any kind of tissue required. Tumors are comparable to non-healing wounds, which use MSCs to help build up the normal tissue that is needed to support the cancer. There is constant remodeling of tissue in tumors, which attracts the stem cells to them.

M.D. Anderson has filed patent applications on a system, which uses human mesenchymal progenitor cells (MPCs). In their novel delivery system, researchers isolate a small quantity of MPC from bone marrow and greatly expand the quantity of those cells in the laboratory. They used an Ad vector to deliver the gene that expresses interferon-β, which can prevent cell reproduction. When turned on, this gene will produce an anticancer effect. When reinfused into the patient through an intravenous injection, the engineered MPCs engraft where the tumor environment is signaling them and will activate the therapeutic gene. Another study demonstrated that when the gene medicine was injected into the carotid artery of mice with human brain cancer, the genes incorporated themselves into the cancer, not into normal brain tissue. These results suggest that gene-modified MPCs can inhibit the growth of leukemias, metastatic tumors of the lungs, and ovarian and brain tumors.

11.12.12 Hematopoietic Stem Cells for Targeted Cancer Gene Therapy

Gene transfer into human HSCs enables enhancement of anticancer immunity as well as increases their resistance to cytotoxic drugs. MDR (multiple drug resistance) gene can be introduced in HSC to reduce chemotoxicity and allow administration of higher doses of chemotherapy. Tumor cell eradication can also be enhanced by genetic modification of chemosensitivity and immunomodulation.

Hematopoietic cells differentiate in steps marked by the acquisition or loss of specific phenotypic characteristics. It is possible to efficiently purify cells with characteristics of HSCs by using techniques combining cytokine stimulation with antimetabolic treatments. Cytokines have also been shown to improve gene transfer to human HSCs.

Gene marking of HSCs provides no direct therapeutic advantage to patients, but information gained from these studies helps to improve the outcome of therapies that incorporate autologous HSC transplantation as a device for eradicating tumors. Several clinical studies have used retroviral gene-marked autologous blood or bone marrow cells to examine the engraftment of HSCs following high-dose chemotherapy and to determine the contribution of tumor cells contaminating the autograft to relapse of the disease. Double gene marking has been used to monitor bone marrow purging and to compare the long-term reconstitution from different populations of hematopoietic progenitor cells.

Angiogenic tumor vessels are promising targets for the activity and the selective delivery of cancer therapeutics. The bone marrow contributes different cell types to the tumor stroma, including hematopoietic cells and vascular endothelial cells. Therefore, transplantation of genetically modified bone marrow progenitors may represent a method for the transport of gene therapy to tumors.

11.12.13 Nanomagnets for Targeted Cell-Based Cancer Gene Therapy

The impact of gene therapy on cancer cells can be enhanced by "magnetic targeting," i.e., inserting nanomagnets into cells carrying genes so that the number of cells successfully reaching and invading cancer can be increased. Systemic administration of such "magnetic" monocytes to mice bearing solid tumors led to a marked increase in their extravasation into the tumor in the presence of an external magnet. Further studies are exploring the effectiveness of magnetic targeting in delivering a variety of cancer-fighting genes, including ones that could stop the spread of tumors. Iron oxide-based nanomagnets, besides their role in diagnosis and hyperthermic therapy of cancer, can be effective for targeted delivery of therapeutic anticancer genes (Meng Lin et al. 2010).

11.12.14 Nanoparticles for Targeted Site-Specific Delivery of Anticancer Genes

Nanoparticles have been used for cancer drug delivery. Some nanoparticles such as dendrimers have multiple points of attachment on a surface for ligands that can attach to multiple disease-specific receptors on targeted cell surfaces. This payload carried by a nanoparticle can be encapsulated within the nanoparticle or attached to the surface and can include diagnostics, therapeutic drugs, DNA, and radiation. A synthetic vector system based on polypropylenimine dendrimers has the desired properties of a systemic delivery vehicle and mediates efficient transgene expression in tumors after intravenous administration (Dufes et al. 2005). Specifically, the

systemic injection of dendrimer nanoparticles containing a TNF-α expression plasmid regulated by telomerase gene promoters (hTR and hTERT) leads to transgene expression, regression of remote xenograft murine tumors, and long-term survival of up to 100 % of the animals. The combination of pharmacologically active synthetic transfection agent and transcriptionally targeted antitumor gene creates an efficacious gene medicine for the systemic treatment of experimental solid tumors. The promising results of these experiments could make it possible to treat inaccessible tumors in humans using gene therapy in the future. This new treatment can selectively target cancer cells, without causing damage to surrounding healthy cells.

11.12.15 Tumor-Targeted Gene Therapy by Receptor-Mediated Endocytosis

Surface-shielded DNA delivery systems have been synthesized with viruslike characteristics that target gene expression into distant tumor tissues. Polyethylenimine (PEI)/DNA complexes ("polyplexes") conjugated with the cell-binding ligand transferrin (Tf) or epidermal growth factor (EGF) have been used to achieve receptor-mediated endocytosis in one study. The surface charge of the complexes can be masked by covalently linking PEI to polyethylene glycol (PEG). Three alternatives for generating these surface-shielded formulations have been utilized, attaching ligand and PEG molecules to PEI either before or after DNA complex formation. The stabilized formulations could be ultra-concentrated, stored frozen, and applied systemically after thawing. Intravenous injections of Tf-PEG-coated polyplexes have been used successfully for gene transfer targeting human carcinoma xenografts in SCID mice. Repeated systemic administration of Tf-PEG-PEI/DNA complexes encoding TNF-α into tumor-bearing mice induces tumor necrosis and inhibition of tumor growth.

11.13 Virus-Mediated Oncolysis

Virotherapy (therapeutic use of viruses) has been employed in oncology. Current design of genetically engineered viruses for selective destruction of cancer cells is based on the observation that attenuated viruses replicate better in tumor cells than in normal cells. Genes that are essential for virus replication in normal tissues are deleted crippling the virus in normal tissues and making it safer. These same genes, however, are expendable in cancer cells because cancer has already activated the cellular targets or homologues of the viral gene (i.e., the cancer cell has done the job for the viral gene already). This is targeting cancer's Achilles' heel since these same genetic changes in cancer that support replication and targeting of viruses are also critical to cancer progression itself.

The ideal virus, however, is one that can infect only cancer cells by virtue of altered host range. Such a virus can be made more robust than the highly attenuated

viruses used in clinical trials. Viruses can selectively infect and kill cancer cells by bursting them open while leaving the normal cells intact. Measles virus (MV) has an oncolytic as well as an immunotherapeutic effect on cancer. Some viruses such as HSV may also deliver genes that make cancer cells more susceptible to chemotherapy. There is a synergy between oncolytic viral therapy and conventional radiotherapy or chemotherapy. However, a single-agent approach may not be adequate to completely eradicate cancer in a patient because most cancers arise from abnormalities in multiple genetic and signal transduction pathways.

11.13.1 Cytokine-Induced Killer Cells for Delivery of an Oncolytic Virus

A limitation of oncolytic virus therapy alone is that effective levels of the virus cannot be maintained easily due to accelerated elimination by the immune system. Cytokine-induced killer (CIK) cells are immune cells that are harvested from a cancer patient's blood, expanded, and administered to the patient, where they traffic to the tumor site and attack cancer cells. However, the tumor-killing potential is only moderate in most cases. A method has been developed for combining these two moderately effective therapeutic approaches to significantly increase anticancer effectiveness. Immediately prior to administering to the host, CIK cells were preinfected with an oncolytic vaccinia virus (Thorne et al. 2006). Whole-body optical imaging, also known as in vivo bioluminescence imaging, revealed that the infected CIK cells, which retain their typical killing profile, reach the tumor. Viral replication and release at the site of the cancer result in systemic delivery of the virus followed by a vigorous anticancer effect. Vaccinia-infected tumor cells proved to be more susceptible to CIK cell killing than noninfected CIK cells. Using several in vivo tumor mouse models—immunocompetent and immunodeficient—the authors have shown that (1) the cells can deliver virus to the tumor with an improved pharmacokinetic profile, (2) the infected cells are still capable of trafficking to the tumor, and (3) this therapeutic approach is substantially more effective at destroying tumors than either of the single approaches used alone (or than both therapies used together but without preinfecting the CIK cells). In one set of results, the method resulted in complete recovery for an entire group of mice with ovarian tumors. In a second group of mice with breast tumors, 75 % recovered completely. As a gene-based cancer therapy, it can be applied to solid tumors as well as lymphoma and can be combined with conventional cancer treatment.

11.13.2 Monitoring of Viral-Mediated Oncolysis by PET

The safety and efficacy of viral oncolysis depend on selective and robust viral replication in cancer cells rather than in normal cells. Methods to detect and quantify viral replication in tissues have relied on organ sampling for molecular analyses.

Preclinical and clinical studies of viral oncolysis will benefit significantly from development of a noninvasive method to repetitively measure viral replication. PET enables in vivo detection of HSV-1 replication in tumor cells using 9-(4-[^{18}F]-fluoro-3-[hydroxymethyl]butyl)guanine (^{18F}FHBG) as the substrate for HSV-tk (Kuruppu et al. 2007). As expected, phosphorylated ^{18F}FHBG is initially trapped within HSV-1-infected tumor cells and is detectable as early as 2 h following virus administration. MicroPET images reveal that ^{18F}FHBG accumulation in HSV-1-infected tumors peaks at 6 h. However, despite progressive accumulation of HSV-1 titers and HSV-tk protein in the tumor as viral oncolysis proceeds, tumor cell degradation resulting from viral oncolysis increases over time, which limits intracellular retention of ^{18F}FHBG. These observations have important consequences with regard to strategies to use ^{18F}FHBG PET for monitoring sites of HSV-tk expression during viral oncolysis.

11.13.3 Oncolytic Adenoviruses

Several genetically engineered adenoviruses (Ad) are used to destroy tumors. These vectors are designed to replicate specifically in solid tumors in cancer patients, to destroy the tumors, and to have minimal side effects. In particular, the vectors are designed such that they cannot harm normal noncancerous tissues in the body.

In principle, conditionally replication-competent Ad that induces tumor oncolysis through cancer-specific replication holds promise for cancer therapy. Based on these considerations, a novel class of cancer destroying Ad has been produced, cancer terminator virus, in which cancer-specific replication is controlled by the PEG-3 promoter and replicating viruses produce a second transgene encoding an apoptosis-inducing and immunomodulatory cytokine, either melanoma differentiation-associated gene-7/interleukin-24 (mda-7/IL-24) or IFN-γ (Das et al. 2012). The current cancer terminator viruses represent the next generation of therapeutic viruses that enable replication uniquely in cancer cells with simultaneous production of immune modulating and toxic genes. These viruses effectively eliminate primary tumors and metastases without harming normal cells or tissues. This dual cancer-specific targeting strategy provides an effective approach for eradicating both primary tumors and metastatic disease.

Ad-mediated delivery of mda-7/IL-24 not only inhibits prostate cancer cell growth but also forces overexpression of bcl-2 or bcl-xL renders prostate cancer cells resistant to Ad.mda-7. A conditionally replication-competent adenovirus has been constructed in which expression of the adenoviral E1A gene, necessary for replication, is driven by the cancer-specific promoter of PEG-3 and which simultaneously expresses mda-7/IL-24 in the E3 region of the adenovirus (Ad.PEG-E1A-mda-7) (Sarkar et al. 2007). Infection of normal prostate epithelial cells and parental and bcl-2- or bcl-xL-overexpressing prostate cancer cells with this terminator virus confirmed cancer cell-selective adenoviral replication, mda-7/IL-24 expression,

growth inhibition, and apoptosis induction. Injection of terminator virus into athymic nude mice completely eradicated not only primary tumors but also distant tumors. These findings indicate potential therapeutic applications for advanced prostate cancer patients with metastatic disease.

VirRx Inc. has developed three potential adenovirus type 5 (Ad5)-based replication-competent cancer gene therapy vectors named KD1, KD3, and VRX-007. All three vectors overexpress an Ad5 protein named Adenovirus Death Protein (ADP). ADP is required for efficient lysis of Ad5-infected cells and spread of virus from cell to cell, and thus its overexpression increases the oncolytic activity of the vectors. KD3 has three key features, i.e., a mutation in the "E1A" gene, overexpression of an adenovirus protein, and deletion of a group of genes named E3. KD1 is similar to KD3. The E1A protein, encoded by the E1A gene, has two major functions in adenovirus infections. One function is to turn on expression of all other adenovirus genes so that adenoviral proteins are made and virions can form. The other function is to deregulate the cell cycle of quiescent cells so that viral gene expression and DNA replication can occur efficiently. The "cell deregulation" portion of the E1A protein forces the cell to make the enzymes required to synthesize DNA. Also, the cell becomes dedifferentiated, assuming a state that is conducive to adenoviral DNA replication. After this occurs, viral genes are expressed well and the viral genome can replicate.

Although several vectors have been developed to express cytotoxic genes via tumor-specific promoters or to selectively replicate in tumor cells, most are taken up and expressed by just a few targeted tumor cells. By contrast, the blood-borne Sindbis viral vectors systemically and specifically infect tumor cells throughout the body without adverse effects. A single intraperitoneal treatment enables the vectors to target most tumor cells, as demonstrated by immunohistochemistry, without infecting normal cells. The virus has also been engineered to carry the IL-12 gene, which has proven to be a good cancer killer but is also toxic to normal tissues. By putting the IL-12 gene into Sindbis, the gene is activated only after it had been carried into cancerous tissues by the virus. The combination is even more effective than Sindbis alone at killing tumors, and it does not poison surrounding areas. It is not known exactly why the virus prefers to bind to tumor cells. But Sindbis enters cells through a receptor for laminin, a substance that helps to glue cells together to form tissues, and tumor cells tend to overexpress this receptor. Because the tumor cells are far more likely than healthy cells to have free laminin receptors on their surfaces, they are more likely to take up the virus.

Ad is one of the most commonly used vectors for gene therapy and two products, Gendicine and Oncorine, have already been approved for treatment of cancer in China. An intriguing aspect of oncolytic adenoviruses is that by their very nature they potently stimulate multiple arms of the immune system. Thus, combined tumor killing via oncolysis and inherent immunostimulatory properties make these viruses in situ tumor vaccines (Cerullo et al. 2012). Oncorine (H101, Shanghai Sunway Biotech Co.), a genetically modified Ad, is injected into the tumor to infect and lyse the tumor cells. Patients develop fever that can induce the production of heat shock protein (HSP), which works as a chaperon and facilitates the induction of

tumor-specific cytotoxic lymphocytes. External heating of the tumor can also initiate thermally induced HSP synthesis.

Ads are attractive for gene therapy because they are relatively innocuous, easy to produce in large quantities, genetically stable, and easy to manipulate. Several of these have been constructed and tested in preclinical and clinical experiments. Oncolytic Ads proved to be remarkably safe with no dose-limiting toxicity were observed in any clinical trial, and the maximum tolerated dose was not reached. Currently, the major challenge for researchers is to increase the efficacy of vectors and to incorporate oncolytic virotherapy into existing treatment protocols (Toth et al. 2010).

11.13.4 Oncolytic HSV

A recombinant HSV 1 (R5111) has been constructed in which the capacity to bind heparan sulfate is disabled and which contained a chimeric IL-13-glycoprotein D that enables the virus to infect cells expressing the IL-13α2 receptor (IL-13Rα2) commonly found on the surface of malignant glioblastomas or high-grade astrocytomas. The recombinant R5111 is able to enter and infect cells via the interaction of the chimeric glycoprotein D with IL-13Rα2, but the virus retains the capacity to bind and replicate in cells expressing the natural viral receptors HveA or nectin-1. Now a recombinant virus (R5141) has been constructed, which can only enter and replicate in cells that express the IL-13Rα2, and does not depend on endocytosis to infect cells (Zhou and Roizman 2006). It does not infect cells expressing HveA or nectin-1 receptors or cells expressing IL-13Rα2 that had been exposed to soluble IL-13 before infection. Thus the host range of HSVs can be altered by genetic manipulation to specifically target cancer cells.

VIRTTU Biologics uses HSV1716 (SEPREHVIR®), a modified version of HSV, in which the gene encoding ICP34.5 has been deleted. HSV1716 is unable to replicate in most cells in the body including brain cells but replicates efficiently in tumor cells. HSV1716 is self-generating, and the effective dose increases rather than decreases, enhancing the therapeutic potential.

11.13.5 Oncolytic Measles Viruses

Oncolytic MVs derived from the live attenuated vaccine strain have been engineered for increased anticancer activity and are currently under investigation in clinical phase I trials. Approaches with other viral vectors have shown that insertion of immunomodulatory transgenes enhances the therapeutic potency. Therapeutic efficacy and adaptive immune response of an engineered MV expressing the cytokine GM-CSF have been evaluated in the context of oncolysis in the immunocompetent murine colon adenocarcinoma model MC38cea, which express the human CEA (Grossardt et al. 2013). Intratumoral administration of MV-GM-CSF significantly

delayed tumor progression and prolonged median overall survival compared with control virus-treated mice. More than one-third of mice treated with MV-GM-CSF showed complete tumor remission and rejected successive tumor reengraftment, demonstrating robust long-term protection. An enhanced cell-mediated tumor-specific immune response could be detected by lactate dehydrogenase assay and interferon-γ ELISA. Furthermore, MV-GM-CSF treatment correlated with increased abundance of CD3+ TILs. These findings indicate the potential of oncolytic MV-GM-CSF as an effective therapeutic cancer vaccine that actively recruits adaptive immune responses for enhanced therapeutic impact and tumor elimination. Thus, the treatment benefit of this combined immunovirotherapy approach has direct implications for future clinical trials.

11.13.6 Oncolytic Vaccinia Virus

There are two products in clinical trials in this category. JX-594 (Jennerex Biotherapeutics' Pexa-Vec) is a vaccinia virus that has been engineered to infect, multiply in, and kill cancer cells while leaving neighboring healthy cells unharmed. In addition to direct killing of cancer cells by viral replication, JX-594 expresses a transgene GM-CSF which stimulates the immune system to recognize and destroy the cancer cells, thereby attacking tumors through multiple mechanisms of action. The results of a phase I study for treatment of metastatic melanoma showed good tolerability and evidence of anticancer effects. A randomized phase II trial of JX-594 showed oncolytic and immunotherapeutic mechanism of action, tumor responses, and dose-related survival in individuals with hepatocellular carcinoma (Heo et al. 2013). JX-594 treatment caused disruption of tumor perfusion as early as 5 days in both VEGF receptor inhibitor-naïve and inhibitor-refractory patients (Breitbach et al. 2013). This platform technology opens up the possibility of multifunctional engineered vaccinia products that selectively target and infect tumor-associated endothelial cells, as well as cancer cells, resulting in transgene expression, vasculature disruption, and tumor destruction in humans systemically.

The second product is GL-ONC1 (Genelux Corporation)—a genetically stable oncolytic virus designed to locate, enter, colonize, and destroy cancer cells without harming healthy tissues or organs. GL-ONC1 is based on vaccinia virus (Lister strain), which was used safely in millions of people as the vaccine against smallpox. Scientists at Genelux have modified this virus to increase its safety, tumor selectivity, and antitumor activity without limiting its ability to replicate in cancer cells. It is currently in phase I/II clinical trials for various cancers.

11.13.7 Oncolytic Vesicular Stomatitis Virus

Vesicular stomatitis virus (VSV) has potential both as an immunization vector and as an oncolytic virus. Safety profile of the virus is an important concern for both

applications. A highly attenuated virus, VSV-12′GFP, was generated by adding two reporter genes to the 3′ end of the VSV genome and displayed the slowest growth kinetics (van den Pol and Davis 2013). The mechanism of attenuation appears to be due to reduced expression of VSV genes downstream of the reporter genes, as suggested by a 10.4-fold reduction in L-protein RNA transcript. Despite attenuation, VSV-12′GFP was highly effective in generating an immune response and showed a greater level of transfection of human cancer cells (glioma and melanoma) than of normal cells, and this effect was magnified in glioma by interferon application, indicating selective oncolysis. Intravenous VSV-12′GFP selectively infected human gliomas implanted in SCID mice subcutaneously or intracranially. Intratumoral injection of tumors with VSV-12′GFP dramatically suppressed tumor growth and enhanced survival. Together these data suggest this recombinant virus merits further study for its oncolytic and vaccine potential.

11.13.8 Concluding Remarks on Oncolytic Gene Therapy

Current therapies only use tumor-selective viruses to guarantee the exclusive spreading of viruses in cancer cells and to avoid damage to healthy surrounding tissue. One problem is that tumors are a mixture of degenerated cells and normal cells, which restrict the intratumoral spreading of the virus leading to therapy resistance. This drawback of oncolytic therapy can be overcome by a tumor-specific replicating adenovirus which expresses adapter proteins on its surface. The replication of this virus only in tumor cells is assured by a triple-saved effective p53-mediated transcriptional repression. The structure of the surface, the adapter protein, allows the virus a receptor-independent uptake into all cells of the tumor. Such independent receptor infections are faster and more effective: the virus propagates better and effectively destroys the tumor. In contrast to conventional tumor-selective oncolysis, the new approach focuses on the tumor and its marginal zones, and the spread of viruses to other parts of the body is significantly reduced. This strategy can be applied in a wider range of oncolytic gene therapy. Advantages of tumor-specific viral oncolysis are:

- Significant improvement of primary infection rate.
- Receptor-independent tumor infection.
- Improved therapeutic effect of viral oncolysis in tumors with existing receptors.
- Increased efficacy of oncolysis in combination with chemotherapy or cytokines.
- Virus replication is confined to cancer cells sparing the healthy surrounding tissue.

11.13.9 Companies Developing Oncolytic Viruses

Companies developing oncolytic viruses are listed in Table 11.5.

Table 11.5 Companies developing oncolytic viruses

Company	Technology/product/mechanism of action	Application/stage
Amgen	OncoVEX GM-CSF (talminogene laherparepvec): engineered HSV	Metastatic melanoma/ phase III
Daewoong	Oncolytic adenoviral gene therapy	Solid cancers
Genelux Corporation	GL-ONC1 is a highly selective, attenuated vaccinia virus-based oncolytic therapy combined with a diagnostic for monitoring	Phase I/II trials in various cancers
Jennerex Biotherapeutics	Pexa-Vec (JX-594): engineered vaccinia virus can infect, multiply in, and kill cancer cells but spares healthy cells	Melanoma, liver cancer, and solid tumors/phase II
Oncolytics Biotech	REOLYSIN, a proprietary formulation of reovirus, can freely replicate and kill tumor cells with an activated Ras pathway	Prostate and brain cancers/phase II
Oncos Therapeutics	CGTG-102, based on the serotype 5 Ad, is potent, selective, and stimulates anticancer immune response	Solid cancer/phase I
Shanghai Sunway Biotech	Licensed ONYX-015 for therapeutic use as H101 genetically modified oncolytic adenovirus with E1B-55 kDa deletion in the viral genome, which gene is needed for the virus to effectively replicate in cells	Solid tumors/used in China clinically. Not approved in the USA or Europe
Viralytics Ltd.	Cavatak: intratumoral injection of a preparation of wild-type Coxsackievirus A2 oncolyses by targeting melanoma cells that overexpress the molecules ICAM-1 and/or DAF	Malignant melanoma/ phase II
VirRx Inc.	Ad 5-based replication-competent cancer gene therapy vectors overexpressing Adenovirus Death Protein (ADP)	Lung cancer/preclinical
VIRTTU Biologics	SEPREHVIR® (HSV1716): genetically modified HSV that selectively kill tumor cells. Can also deliver anticancer agents	Several solid tumors/phase I/II

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11.14 Apoptotic Approach to Improve Cancer Gene Therapy

Advances in our understanding of the mechanisms by which tumor cells detect drug-induced DNA damage leading to apoptotic death have aided in the design of novel, potentially more selective strategies for cancer treatment. Several of these strategies use proapoptotic factors and have shown promise in sensitizing tumor cells to the cytotoxic actions of traditional cancer chemotherapeutic drugs. Although antiapoptotic factors are generally regarded as poor prognostic factors for successful cancer chemotherapy, strategies that use antiapoptotic factors in combination with suicide or other gene therapies can also be considered. The introduction of antiapoptotic factors that act downstream of drug-induced mitochondrial transition delays, but does not block, the ultimate cytotoxic response to cancer

chemotherapeutic drugs that activate a mitochondrial pathway of cell death. Recent studies using the cytochrome P450 prodrug cyclophosphamide exemplify how the antiapoptotic, caspase-inhibitory baculovirus protein p35 can be combined with P450 GDEPT to prolong localized, intratumoral production of cytotoxic drug metabolites without inducing tumor cell drug resistance. This model may be adapted to other gene therapies, including those that target death receptor pathways, to maximize the production of soluble, bystander cytotoxic factors and prodrug metabolites and thereby amplify the therapeutic response.

A specific gene, melanoma differentiation-associated gene-7/interleukin-24 (mda-7/IL-24), displaying cancer-specific apoptosis-inducing properties isolated using this scheme has now come into the limelight as a new gene therapy for divergent cancers. Although the mechanism of cancer cell selectivity of mda-7/IL-24 remains to be delineated, numerous attributes enable this gene as an effective therapy for cancer, including an ability to discriminate between normal and cancer cells, induce apoptosis in diverse tumor cells, promote "bystander" antitumor effects, inhibit tumor growth and angiogenesis in animal models, synergize with radiation, and modulate immune responses. These unique features combined with successful transition into the clinic instill confidence that mda-7/IL-24, as a single or more likely as part of a combinatorial approach, may provide profound therapeutic benefit for cancer patients (Fisher 2005).

11.15 Tumor Suppressor Gene Therapy

Cellular genes that regulate cell growth by counteracting the action of proto-oncogenes are called tumor suppressor genes. Potential sites where these genes might inhibit the development of cancer include cell proliferation, differentiation, and senescence; cell-to-cell communication; and chromosomal stability. Mutations in tumor-suppressing genes cause growth-inhibiting proteins encoded by the genes to disappear, allowing the cell to survive and continue dividing when normally it should not. An excess of oncoproteins and lack of tumor suppressor proteins lead mutant cells to reproduce excessively. p53, Rb, and BRIT1 are examples of tumor suppressor genes, which can form the basis of gene therapy for cancer.

11.15.1 p53 Gene Therapy

p53 is known as a tumor suppressor gene important for maintenance of genomic integrity. p53 is a short-lived protein that is maintained at very low levels in cells. p53 is regulated by MDM2 protein which participates in p53 rapid degradation. The activation and accumulation of p53 is a response to cellular stress such as DNA damage. Activated p53 is a sequence-specific DNA-binding transcription factor, and some target genes of its transcriptional activity are important for cell cycle

arrest or for inducing apoptosis. p53 is also used for cancer therapy by p53 gene therapy when p53 is applied into tumors or by reactivation of mutant p53.

11.15.2 BRIT1 Gene Therapy

A signaling network of molecular checkpoint pathways protects the human genome by detecting DNA damage, initiating repair, and halting division of the damaged cell so that it does not replicate. The gene BRIT1 activates two of these checkpoint pathways: the ATM pathway springs into action in response to damage caused by ionizing radiation, and the ATR pathway responds to DNA damage caused by ultraviolet radiation. BRIT1 is underexpressed in human ovarian, breast, and prostate cancer cell lines, and defects in BRIT1 seem to be a key pathological alteration in cancer initiation and progression (Rai et al. 2006). Disruption of BRIT1 function abolishes DNA damage responses and leads to genomic instability, which fuels the initiation, growth, and spread of cancer. By using siRNA to silence the BRIT1 gene, the scientists shut down both checkpoint pathways in cells exposed to either type of radiation. siRNA was then used to silence the gene in normal human mammary epithelial cells (HMECs), with the result that inactivation of the gene caused chromosomal aberrations in 25 % of cells. Control group HMEC had no cells with chromosomal aberrations. In cells with the gene silenced that were then exposed to ionizing radiation, 80 % of cells had chromosomal aberrations. Reduced BRIT1 expression is also found in advanced epithelial ovarian cancer as well as in prostate cancer tissue compared with noncancerous cells. Thus, BRIT1 may function as a tumor suppressor, and as such, further understanding of its function may form the basis of gene therapy for cancer.

11.16 Nitric Oxide-Based Cancer Gene Therapy

11.16.1 Anticancer Effect of Nitric Oxide Synthase

Nitric oxide (NO), produced by nitric oxide synthase (NOS), is the main mediator of the tumoricidal action of activated macrophages. Inducible nitric oxide synthase (iNOS) gene therapy has been reported to have antitumor effects in several types of cancers and enhances sensitivity to cisplatin. A study has evaluated the effects of cationic liposome (LP)-mediated iNOS gene transfection in enhancing low-dose cisplatin-mediated antitumor effects in lung cancer cell xenograft mouse models (Ye et al. 2013). The results showed that iNOS gene therapy significantly enhanced low-dose cisplatin-mediated inhibition of cell proliferation, invasion, migration, and promotion of cell apoptosis. iNOS gene-mediated enhancement of cisplatin's antitumor effects in lung cancer may be related to the attenuation of

p-mTOR, MMP2, and activation of p53. Thus, the combination treatment with iNOS gene therapy and cisplatin may be a novel and effective therapeutic strategy for lung cancer.

11.16.2 NOS-Based Gene Therapy for Radiosensitization of Cancer

NO is a potent radiosensitizer of tumors, but its clinical use is limited by serious side effects when administered systemically. Gene transfer of the iNOS into colorectal cancer cells has been shown to enhance radiation-induced apoptosis in vitro. Adenoviral gene transfer of iNOS (AdiNOS) into human colorectal cancer cell lines significantly enhances the effects of radiation associated with increased iNOS expression and NO production. The radiosensitizing effects of AdiNOS occur at low infection efficiency, indicating a significant bystander effect. Significant chemosensitization of cisplatin cytotoxicity was observed various cancer cell lines in the presence of NO derived from the overexpression iNOS, which was considered unlikely o be related to p53 status (Adams et al. 2009).

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Chapter 12
Role of RNAi in Cancer

12.1 Introduction

RNA interference (RNAi) is an ancient natural antiviral mechanism that directs silencing of gene expression in a sequence-specific manner. It is one of the technologies for suppression of gene function of which the best known involves the use of antisense oligonucleotides. RNAi involves the use of a double-stranded RNA (dsRNA). Once in the cell, the dsRNAs are processed into short, 21–23 nucleotide dsRNAs termed small interfering RNAs (siRNAs) that are used in a sequence-specific manner to recognize and destroy complementary RNAs. There are several classes of naturally occurring small RNA species, including siRNAs, repeat-associated siRNAs (rasiRNAs), and microRNAs (miRNAs); the last type is described in Chap. 13. Basics of RNAi and details of RNAi-based therapeutics are described in detail in a special report on this topic (Jain 2013).

The cell uses the sequence information in long dsRNA to target a corresponding mRNA for destruction. In the RNAi pathway, long dsRNA is cleaved into siRNAs through action of Dicer, a cellular ribonuclease III. These siRNAs are unwound, and one of the two strands becomes associated with a complex of proteins and the target transcript designated as the RNA-induced silencing complex (RISC), which leads to homologous target RNA destruction. Nearly every animal and plant cell has internal machinery that uses unusual forms of RNA to naturally silence a particular gene. This machinery was evolved to protect cells from hostile genes and to regulate the activity of normal genes during growth and development. Observations of RNAi in the nuclei of human cells raise questions about the extent to which nuclear and cytoplasmic RNAi pathways are shared. By directly visualizing the localization of siRNA in live human cells, it was shown that siRNA either selectively localizes in the cytoplasm or translocates into the nucleus, depending on where the silencing target RNA resides (Berezhna et al. 2006). The results of this study suggest the existence of a mechanism by which the RNAi machinery orchestrates a target-determined localization of the siRNA and the corresponding RNAi activity and also provide evidence for formation of nuclear-programmed active RISCs directly in the nucleus.

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_12,
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12.2 Delivery of RNAi Therapeutics

Delivery of therapeutics to the target issues is an important consideration in RNAi. There are several challenges. Ability to efficiently and stably produce and deliver sufficient amounts of siRNA to the proper target tissues require refinement before this new technology can be tried clinically. Initial in vivo studies reported effective transgene suppression in adult mice by chemically synthesized siRNAs. Several researchers have used plasmid and viral vectors for transcription of short-hairpin RNAs (shRNAs), both in vitro and in vivo. With these expression systems, gene expression is more stably inhibited than with the transient knockdown recorded with chemically synthesized siRNA.

12.2.1 siRNA Delivery Technologies

Successful knockdown of genes requires efficient delivery of siRNAs. Because introduction of long dsRNA also induces components of the interferon response pathway in non-embryonic mammalian cells, RNAi is usually induced by direct introduction of siRNA into cells. The level of knockdown of the target mRNA can be close to 20-fold and last for a few days in dividing cells before mRNA levels begin to recover. Simple addition of naked unmodified siRNAs to the culture media over cells does not result in effective knockdown of the target gene. An siRNA designed to be specific for a promoter can be placed into human cells and silences the promoter if it gets into the nucleus. A nuclear-specific reagent or a lentiviral vector may alter the cell membrane and let the siRNA enter. Various methods of delivery of siRNA are listed in Table 12.1. Those relevant to siRNA delivery for cancer therapy are described in the following text.

Both biological and chemical approaches offer potential for the desired delivery of therapeutic RNAi to the cell cytoplasm or the nucleus. Currently the RNAi-based drugs are delivered into cells using transfection agents or by electroporation and into animals by hydrodynamic methods (loading the animals with a rapid infusion of a volume one-tenth their mass). This brute-force delivery method is thought to promote transfection by causing physical damage to liver cell membranes. Other methods of transfection are by viral vectors or by complexing with cationic lipids. Local administration of siRNA involves direct introduction of siRNA into a particular tissue or organ. There are several reports of successful introduction of siRNA into the eyes, lungs, and brains of experimental animals. These methods demonstrate the therapeutic potential of RNA-based drugs, but their use in human therapeutics is not realistic.

12.2.2 In Vivo Delivery of siRNAs by Synthetic Vectors

Achieving efficient in vivo delivery of siRNA to the appropriate target cell would be a major advance in the use of RNAi in gene function studies and as a therapeutic

Table 12.1 Methods of delivery of siRNA

Delivery of siRNA to mammalian cells in culture
Lipofection: negatively charged siRNAs bind to cationic lipid particles by electrostatic interaction and the complex enters the cell through endocytosis
Delivery of siRNA by use of other chemical methods, e.g., CaP precipitation
TransIT-TKO (Mirus Bio Corporation)
Electroporation
Protein transduction domains
PCR cassettes expressing siRNAs
Delivery of siRNA expression vectors
Delivery of siRNA expression cassettes by viral vectors
In vivo delivery of siRNA
Local administration, e.g., into tumors, intrathecal, intranasal
Systemic administration, e.g., intravenous
Antibody-mediated siRNA delivery targeted to cell surface receptors
Intravascular hemodynamic siRNA delivery, e.g., into portal vein
Delivery of siRNA by synthetic vectors, e.g., lipid vectors
Viral vectors for in vivo delivery of siRNA

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modality. Several techniques are under investigation for this purpose. Conjugation of small molecules to the appropriate sites of RNA drugs can dramatically enhance protein-binding properties and also improve their cell permeation. RNA drugs can also be conjugated with a variety of synthetic vectors equipped with tissue- or cell-specific targeting functionalities to achieve broad in vivo tissue distribution and cellular permeation. Gene silencing by systemic delivery of synthetic siRNAs has been demonstrated in experimental animals.

However, unmodified siRNA can be potent triggers of the innate immune response, particularly when associated with delivery vehicles that facilitate intracellular uptake. This represents a significant barrier to the therapeutic development of siRNA due to toxicity and off-target gene effects associated with this inflammatory response. Immune stimulation by synthetic siRNA can be completely abrogated by selective incorporation of 2′-*O*-methyl (2′OMe) uridine or guanosine nucleosides into one strand of the siRNA duplex (Judge et al. 2006). These noninflammatory siRNAs, containing less than 20 % modified nucleotides, can be readily generated without disrupting their gene-silencing activity. Coupled with an effective systemic delivery vehicle, 2′OMe-modified siRNA targeting apolipoprotein B (apoB) was shown to mediate potent silencing of its target mRNA, causing significant decreases in serum apoB and cholesterol. This is achieved at therapeutically viable siRNA doses without cytokine induction, toxicity, or off-target effects associated with the use of unmodified siRNA. This approach to siRNA design and delivery should prove widely applicable and represents an important step in advancing synthetic siRNA into a broad range of therapeutic areas.

12.2.3 Intracellular Delivery of siRNAs

One stumbling block to successfully administering RNAi-based medicines has been finding a way to shield siRNA until it reaches its intracellular target. In their natural form, siRNAs are quickly cleared from the bloodstream. Even if they reach their intended destination, they are engulfed by the cell's endosomes. The cellular uptake of siRNA represents a major technical hurdle for the biologic effectiveness and therapeutic success in vivo. Subsequent to cellular delivery it is crucial to direct siRNA to the cellular location where it enters the RNAi pathway. Functionally active siRNA represents a minor fraction, i.e., 1 % of total siRNA inside a given target cell. Exploiting possibilities of steering intracellular release or trafficking of siRNA has the potential of substantially increasing the biological activity of siRNA. Several methods have been investigated for this purpose. Some of these are described here.

12.2.4 Delivery of siRNAs with Aptamer–siRNA Chimeras

Aptamers are single-strand oligonucleotides, which can bind to a given ligand with high affinity and specificity due to their particular 3D structure and thereby antagonize the biological function of the ligand. Aptamers that bind to cell surface proteins are well suited for the targeted delivery of other therapeutics, such as conjugated siRNAs that induce RNAi. Thus, aptamer–siRNA chimeras may offer dual functions, in which the aptamer inhibits a receptor function, while the siRNA internalizes into the cell to target a specific mRNA.

A 5′-NH2-modified aptamer was chemically synthesized and covalently decorated on the surface of a polymer composed of branched polyethyleneimine grafted with polyethylene glycol for co-delivery of shRNA and chemotherapy agents (Zhou et al. 2012). Aptamer–siRNA chimeric RNAs, capable of cell type-specific binding and delivery of functional siRNAs into cells, have been developed (McNamara et al. 2006). The aptamer portion of the chimeras mediates binding to prostate-specific membrane antigen (PSMA), a cell surface receptor overexpressed in prostate cancer cells and tumor vascular endothelium, whereas the siRNA portion targets the expression of survival genes. When applied to cells expressing PSMA, these RNAs are internalized resulting in depletion of the siRNA target proteins and cell death. In contrast, the chimeras do not bind to or function in cells that do not express PSMA. These reagents also specifically inhibit tumor growth and mediate tumor regression in a mouse model of prostate cancer. The mice showed no side effects from the treatment. These studies demonstrate an approach for targeted delivery of siRNAs with numerous potential applications, including cancer therapeutics. However, much work remains to be done to move the experimental drug into clinical use in humans. The next step is to demonstrate conclusively that the drug can be delivered into the bloodstream and still reach the tumor target without being destroyed by the body or causing adverse side effects.

12.2.5 Nanoparticles for Intracellular Delivery of siRNA

Evaluation of a large library of structurally distinct nanoparticles with cationic cores and variable shells was carried out by using robotic automation. Nanoparticles were combinatorially cross-linked with a diverse library of amines, followed by measurement of molecular weight, diameter, RNA complexation, cellular internalization, and in vitro siRNA and pDNA delivery (Siegwart et al. 2011). Analysis revealed structure–function relationships and beneficial design guidelines. Cross-linkers optimally possessed tertiary dimethylamine or piperazine groups and potential buffering capacity. Covalent cholesterol attachment enabled intracellular delivery in vivo to liver hepatocytes in mice.

Efforts are being made to develop a lipid-based nanocarrier for siRNA delivery aimed at two cell subpopulations within breast tumors: the cancer and the endothelial cells from angiogenic tumor blood vessels. To achieve this goal, the systemically administered F3 peptide, which is specifically internalized by nucleolin overexpressed on both those subpopulations, was used as a targeting moiety (Gomes-da-Silva et al. 2012). This work represents an important contribution toward a nanoparticle with multi-targeting capabilities in breast cancer, at the cellular as well as the molecular level.

12.2.6 Delivery of siRNA-Lipoplexes

Uptake, biodistribution, and in vivo efficacy of siRNA molecules formulated into siRNA-lipoplexes have been studied after single intravenous injection (Santel et al. 2006). The applied formulation is based on complex formation of positively charged liposomes, a mixture of cationic and fusogenic lipids complexed with the negatively charged siRNA. Furthermore, by using siRNA molecules for targeting genes such as CD31 and Tie2, which are specifically expressed in endothelium, downregulation of the corresponding mRNA and protein in vivo could be demonstrated. These findings show the applicability of this nonviral delivery technology for inducing RNAi in the vasculature of mice after systemic application. Further studies showed that siRNA-lipoplexes, but not naked siRNAs, can be delivered to the tumor endothelial cells in vivo (Santel et al. 2006a). In addition, functional intracellular delivery of formulated siRNA targeting the tumor suppressor PTEN was shown in endothelial cells of the liver and tumor. Finally, the therapeutic potential of systemically administered siRNA(CD31)-lipoplexes was established by inhibition of tumor growth in two different xenograft mouse models. These findings corroborate the applicability of liposomal siRNA delivery technology for inducing RNAi to modulate gene expression levels in angiogenesis-dependent processes. In addition, these results advocate CD31 as a promising therapeutic target for antiangiogenic intervention. The study provides a basis for the development of antiangiogenic cancer therapies based on RNAi.

12.2.7 *Transkingdom RNAi Delivery by Genetically Engineered Bacteria*

One method to overcome barrier to siRNA delivery is the use of a nonpathogenic bacterial vector, *E. coli*, to deliver RNAi to target cells with high efficacy. In transkingdom RNAi (tkRNAi) delivery, *E. coli* are engineered to transcribe shRNA from a plasmid (TRIP) containing the invasin gene Inv and the listeriolysin O gene Hly, which is successful in eliciting efficient gene silencing in vitro and in vivo (Nguyen and Fruehauf 2009). tkRNAi extends the portfolio of viral or nonviral strategies, as the genetically modified bacteria themselves produce shRNAs which, upon bacterial infection, become available for the target cells for further processing and induction of RNAi (Aigner 2009). In bacteria-mediated RNAi (bm-RNAi), the bacteria only deliver the shRNA-encoding DNA construct with subsequent transcription of the shRNA in the target cells.

In one study, the tkRNAi approach was used for modulation of the "classical" ABCB1-mediated multidrug resistance (MDR) in human cancer cells (Krühn et al. 2009). Subsequent to treatment with anti-ABCB1 shRNA expression vector bearing *E. coli*, MDR cancer cells showed 45 % less ABCB1 mRNA expression. ABCB1 protein expression levels were reduced to a point at which merely a weak band could be detected. Drug accumulation was enhanced 11-fold, to an extent that it reached 45 % of the levels in nonresistant cells and resistance to daunorubicin was decreased by 40 %. The data provide the proof-of-concept that tkRNAi is suitable for modulation of MDR in human cancer cells. Although the tkRNAi system tested is still being optimized and did not attain the levels of gene silencing seen with conventional siRNAs or virally delivered shRNAs, it has the potential to become a powerful tool for delivery of RNAi effectors for the reversal of cancer MDR in future.

12.2.8 *Nanobiotechnology-Based Delivery of siRNAs*

12.2.8.1 Lipid Nanoparticle-Based Delivery of Anticancer siRNAs

Tekmira Pharmaceuticals' LNP (lipid nanoparticle) is being developed as a novel, safe, and effective anticancer drug directed against PLK (polo-like kinase), a protein involved in cancer cell proliferation. Inhibition of PLK prevents the cancer cell from completing cell division, resulting in cell cycle arrest and cell death. LNPs are particularly well suited for the delivery of siRNA to treat cancer because the LNPs preferentially accumulate within malignant tumors having leaky blood vessels. Once at the target site, LNPs are taken up by tumor cells and the siRNA payload is delivered inside the cell where it reduces expression of the target protein. In preclinical studies, PLK LNPs displayed potent and specific anticancer effects in a variety of cancer models in animals, translating into significant survival benefits even when measured in aggressive liver cancer models. PLK LNP also sensitizes cancer cells to the effects of chemotherapy drugs such as Taxol. Formal safety studies for PLK LNPs have been completed and it is in phase I human clinical trials.

12.2.8.2 Minicells for Targeted Delivery of Nanoscale Anticancer Therapeutics

Indiscriminate drug distribution and severe toxicity of systemic administration of chemotherapeutic agents can be overcome through encapsulation and cancer cell-specific targeting of chemotherapeutics in 400-nm minicells (EnGeneIC Delivery Vehicle). Targeting of minicells via bispecific antibodies to receptors on cancer cell membranes results in endocytosis, intracellular degradation, and drug release. Minicells were shown to specifically and sequentially deliver to tumor xenografts siRNAs or shRNA-encoding plasmids to counteract drug resistance by knocking down a MDR protein (MacDiarmid et al. 2009). Subsequent administration of targeted minicells containing cytotoxic drugs eliminates formerly drug-resistant tumors. The dual sequential treatment, involving minicells loaded with both types of payload, enables complete survival without toxicity in mice with tumor xenografts while involving 1,000-fold less drug, siRNA, and antibody than needed for conventional systemic administration of cancer therapies.

12.2.8.3 Nanoimmunoliposome-Based System for Targeted Delivery of siRNA

A nanoimmunoliposome-based delivery complex (scL) has been developed that will preferentially target and deliver molecules including plasmid DNA and antisense oligonucleotides to tumor cells following systemic administration (Pirollo et al. 2007). This tumor-targeting nanoparticle delivery vehicle can also deliver siRNA to both primary and metastatic disease. The efficiency of this complex has been enhanced by the inclusion of a pH-sensitive histidine–lysine peptide in the complex (scL-HoKC) and by delivery of a modified hybrid (DNA–RNA) anti-HER-2 siRNA molecule. Scanning probe microscopy confirms that this modified complex maintains its nanoscale size. More importantly, this nanoimmunoliposome anti-HER-2 siRNA complex can sensitize human tumor cells to chemotherapeutics, silence the target gene, affect its downstream pathway components in vivo, and significantly inhibit tumor growth in a pancreatic cancer model. This complex has the potential to help translate the potent effects of siRNA into a clinically viable anticancer therapeutic.

A systemically injectable siRNA vehicle, the "wrapsome" (WS) contains siRNA and a cationic lipofection complex in a core that is fully enveloped by a neutral lipid bilayer and hydrophilic polymers (Yagi et al. 2009). WS protects siRNA from enzymatic digestion, providing a long half-life in the systemic circulation. Moreover, siRNA/WS leaks from blood vessels within tumors into the tumor tissue, where it accumulates and is subsequently transfected into the tumor cells. Because the transcription factor KLF5 is known to play a role in tumor angiogenesis, KLF5-siRNA was designed to test the antitumor activity of siRNA/WS. KLF5-siRNA/WS exhibited significant antitumor activity, although neither WS containing control scrambled siRNA nor saline containing KLF5-siRNA affected tumor growth. KLF5-siRNA/WS inhibited Klf5 expression within tumors at both mRNA and

protein levels, significantly reducing angiogenesis, and no significant acute or long-term toxicity was detected. These findings support the idea that siRNA/WS can be used to knock down specific genes within tumors and thereby exert therapeutic effect against cancer.

12.2.8.4 Polymer Nanoparticles for Targeted Delivery of Anticancer siRNA

Calando Pharmaceuticals combines its proprietary technologies in targeted polymeric delivery systems and siRNA design to create effective therapeutics. Cyclodextrin-containing polymers form the foundation for a two-part siRNA delivery system. The first component is a linear, cyclodextrin-containing polycation that, when mixed with siRNA, binds to the anionic "backbone" of the siRNA. The polymer and siRNA self-assemble into nanoparticles of approximately 50 nm in diameter that fully protect the siRNA from nuclease degradation in serum. The cyclodextrin in the polymer enables the surface of the particles to be decorated by stabilizing agents and targeting ligands.

CALAA01 employs a novel nanoparticle delivery system containing nonchemically modified siRNA and a transferrin (Tf) protein-targeting agent formulated with Calando's RONDEL™ (RNA/Oligonucleotide Nanoparticle Delivery). The effects of administering escalating, intravenous (IV) doses of targeted nanoparticles CALAA01 targeting the M2 subunit of ribonucleotide reductase (RRM2) to nonhuman primates have been studied (Heidel et al. 2007a). The data show that multiple, systemic doses of targeted nanoparticles containing nonchemically modified siRNA can safely be administered to nonhuman primates. Further studies have shown that CALAA01 exhibits significant antiproliferative activity in cancer cells of varying human type and species (mouse, rat, monkey); these findings suggest that this duplex is a promising candidate for therapeutic development (Heidel et al. 2007b). CALAA01 is in phase I clinical trials.

12.2.8.5 RNA Nanotechnology for Delivery of Cancer Therapeutics

RNA can be used as a building block for bottom-up assembly in nanotechnology. RNA has immense promise as a therapeutic agent against cancer, but the problem has been to have an efficient system to bring multiple therapeutic agents directly into specific cancer cells where they can perform different tasks. The 25-nm RNA nanoparticles enable repeated long-term administration and avoid the problems of short retention time of small molecules and the difficulties in the delivery of particles larger than 100 nm. Scientists at Purdue University have used these nanoparticles, which are assembled from three short pieces of RNA and resemble miniature triangles. The microscopic particles possess both the right size to gain entry into cells and the right structure to carry other therapeutic strands of RNA inside with

them, where they are able to halt viral growth or cancer's progress. RNA molecules come in many variant forms, and the one mimicked from the PHI29 virus—called pRNA—also can be linked to other types of RNA to form longer, hybrid strands with properties that could be assigned. Incubation of cancer with the pRNA dimer, one subunit of which harbored the receptor-binding moiety and the other harboring the gene-silencing molecule, resulted in their binding and entry into the cells and subsequent silencing of anti-/proapoptotic genes. The chimeric pRNA complex was found to be processed into functional double-stranded siRNA by Dicer (RNA-specific endonuclease). Animal trials confirmed the suppression of tumorigenicity of cancer cells by ex vivo delivery (Guo et al. 2005).

RNA nanotechnology has been used to engineer both therapeutic siRNA and a receptor-binding RNA aptamer into individual pRNAs of phi29's motor (Khaled et al. 2005). The RNA building block harboring siRNA or other therapeutic molecules was fabricated subsequently into a trimer through the interaction of engineered right and left interlocking RNA loops. The incubation of the protein-free nanoscale particles containing the receptor-binding aptamer or other ligands results in the binding and co-entry of the trivalent therapeutic particles into cells, subsequently modulating the apoptosis of cancer cells and leukemia model lymphocytes in cell culture and animal trials. The use of such antigenicity-free 20–40-nm particles holds promise for the repeated long-term treatment of chronic diseases.

12.2.8.6 Chitosan-Coated Nanoparticles for siRNA Delivery

Overexpression of RhoA in cancer indicates a poor prognosis because of increased tumor cell proliferation and invasion and tumor angiogenesis. Anti-RhoA siRNA inhibits aggressive breast cancer more effectively than conventional blockers of Rho-mediated signaling pathways. A study reports the efficacy and lack of toxicity of intravenously administered encapsulated anti-RhoA siRNA in chitosan-coated polyisohexylcyanoacrylate (PIHCA) nanoparticles in xenografted aggressive breast cancers (Pille et al. 2006). The siRNA treatment inhibited the growth of tumors by 90 % and necrotic areas were observed in tumors, resulting from angiogenesis inhibition. In addition, this therapy was found to be devoid of toxic effects. Because of its efficacy and the absence of toxicity, it is suggested that this strategy of anti-RhoA siRNA holds significant promise for the treatment of aggressive cancers.

Chitosan-coated poly(isobutylcyanoacrylate) nanoparticles have been used to deliver siRNA with a complementary sequence to the fusion oncogene ret/PTC1 to inhibit this oncogene in a model of papillary thyroid carcinoma cells (de Martimprey et al. 2008). This siRNA sequence has then been validated by an shRNA approach using the same sequence. Furthermore, the high ret/PTC1 inhibition has triggered a phenotypic reversion of the transformed cells. Reduction of size of the chitosan-decorated nanoparticles has enabled the protection of ret/PTC1 siRNA from in vivo degradation, leading to significant tumor growth inhibition after intratumoral administration.

12.2.8.7 Nanosized Liposomes for Delivery of siRNA

siRNA incorporated into the neutral nanosized liposome 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) has been used for efficient in vivo siRNA delivery. Getting the siRNA to the targeted protein tumor cells, focal adhesion kinase (FAK), is difficult as it is located inside the cell, rather than on the cell surface where most proteins targeted by cancer drugs are found. FAK, which is difficult to target with a drug, can be attacked with the liposomal siRNA approach, which penetrates deeply into the tumor (Halder et al. 2006). Mice infected with three human ovarian cancer cell lines derived from women with advanced cancer were treated with liposomes that contained either the FAK siRNA, a control siRNA, or were empty. Some mice received siRNA-liposomes plus the chemotherapy docetaxel. Mice receiving the FAK-silencing liposome had reductions in mean tumor weight ranging from 44 to 72 % compared with mice in the control groups. Combining the FAK-silencing liposome with docetaxel boosted tumor weight reduction to the 94–98 % range. In addition to its anticancer effect, the therapeutic liposome also had an antiangiogenic effect when combined with chemotherapy. By inducing apoptosis among blood vessel cells, the treatment steeply reduced the number of small blood vessels feeding the tumor, cut the percentage of proliferating tumor cells, and increased cell suicide among cancer cells. Two advantages of this approach are as follows:

1. The FAK-targeting liposome ranges between 65 and 125 nm in diameter. Blood vessels that serve tumors are more porous than normal blood vessels, with pores of 100–780 nm wide. The liposomes do not enter the normal blood vessel, whose pores are 2 nm or less in diameter.
2. The liposome DOPC has no electrical charge. Its neutrality provides an advantage over positively or negatively charged liposomes when it comes to binding with and penetrating cells.

These studies show the feasibility of siRNA as a clinically applicable therapeutic modality. The next step for the FAK siRNA-DOPC liposome is toxicity testing. In addition to ovarian cancer, FAK is overexpressed in colon, breast, thyroid, and head and neck cancers.

12.2.9 Drug Delivery Issues in Managing Cancer by RNAi Approach

For targeted delivery to solid tumors, RNAi is considerably behind antisense DNA. In vitro RNAi specificity for mRNA is very high, but it is not certain how it is going to perform therapeutically. Although siRNA delivery in vivo is a challenging problem, stable expression of siRNA, which targets oncogenic fusion genes, may potentiate the effects of conventional therapy for hematologic malignancies. Most studies of RNAi in cancer involve leukemia, which produces no solid tumors and delivery to the tumor is less of a problem. However, there is need for progress in delivery techniques. To get the RNAi molecule to express in the center of solid tumors is

probably going to be somewhat tricky. The surgeons may still have to get rid of the bulk of the tumor and RNAi could help clear up the residues. Several technologies have been used to improve siRNA delivery in cancer. These include hybrid RNA–DNA molecules, viral vectors, and nanotechnology.

Because of the difficulties in delivering a large amount of siRNA to cancer cells and the short half-life of siRNA, it is important to choose an efficient delivery system for transduction of siRNA into target cells. Oncolytic adenovirus offers a better platform by virtue of its high transfection efficiency and selective replication in cancer cells (Pei et al. 2010). There is synergism between oncolytic adenovirus and siRNA antitumor responses with significantly enhanced antitumor effect through gene knockdown and viral oncolysis.

shRNA, which stably expresses siRNA in target cells, has potential therapeutic use for inhibiting cancer cells where aberrant expression of certain mRNAs is the culprit. Although there are data showing that shRNA can be used in mice, its translation into application for human cancer therapy is still far away because of serious problems involving biodistribution and clearance of nanoparticles following systemic delivery of shRNA-expressing vectors (Wang et al. 2009).

12.3 Therapeutic Applications of RNAi in Oncology

There is an extraordinary opportunity for development of RNAi therapeutics with the availability of genome sequencing data. RNAi has already been successfully used to suppress dominant disease genes in vitro and in vivo; in some cases, this suppression has been allele-specific, silencing the disease-causing allele while maintaining expression of the normal allele.

12.3.1 Impact of RNAi Research on Oncology

Examples of various in vivo demonstrations of RNAi therapeutic efficacy in animal models of human cancers are shown in Table 12.2.

Table 12.2 In vivo RNAi therapeutic efficacy in animal models of human cancers

Cancer type	Target gene	RNAi formulation	Delivery route
Germ cell tumor	FGF-4	siRNA/atelocollagen complex	Intratumoral
Small-cell lung cancer	Skp-2	siRNA/adenoviral vector	Intratumoral
Pancreatic adenocarcinoma	CEACAM6	siRNA	Intravenous/ hydrodynamic
Glioblastoma	MMP-9 + cathepsin B	shRNA/plasmid DNA	Intratumoral

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CEACAM6 carcinoembryonic antigen-related cell adhesion molecule 6, *FGF-4* fibroblast growth factor 4, *MMP* matrix metalloproteinase, *Skp-2* S-phase kinase-associated protein

The aim in cancer is to inhibit expression of mutated proteins that cause cancer cells to proliferate. However, there is a disparity between achieving such results in vitro and in a whole animal or patient. The challenge is to translate this powerful technology into clinical practice of oncology.

RNAi has the potential to impact on cancer research in three ways:

1. Disruption of posttranslational gene-silencing mechanisms plays a direct role in development of cancer associated with impaired expression of tumor suppressor genes.
2. RNAi has been shown to control tumor cell growth in vitro. siRNA or plasmids expressing sequences processed to siRNA could provide an exciting new therapeutic modality for treating cancer.
3. RNAi has been used in determining the functional role of genes associated with cancer development and response to treatment.

Several studies have been conducted to determine the role of RNAi in cancer. Findings of one study indicate that levels of Dicer and Drosha mRNA, two proteins that are vital to a cell's gene-silencing machinery, have associations with outcomes in patients with ovarian cancer (Merritt et al. 2008). Mutational analyses of genomic DNA from the Dicer and Drosha genes were performed in a subgroup of ovarian cancer specimens. Dicer-dependent functional assays, performed by means of in vitro transfection with siRNA and shRNA, indicated that gene silencing with shRNA, but not siRNA, may be impaired in cells with low Dicer expression. The study shows that women with high levels of Dicer and Drosha had a median survival of 11 years, whereas for those with low levels of both proteins, median survival was 2.66 years. Analysis of gene expression data in groups of lung and breast cancer patients revealed similar associations with patient survival.

Some of the most dynamic and exciting applications of siRNA are in cancer research. Functional validation of tumorigenic genes in cell culture and animal tumor models requires effective siRNA delivery systems, efficiency of siRNA agents compared with antisense oligonucleotides, and efforts for potential therapeutic development. Along with the rapidly growing literature on using siRNA as a functional genomic tool, there is emerging evidence that siRNA may represent a novel therapeutic modality for cancer treatment when optimized local and systemic delivery systems are available. Potency and specificity of gene silencing are the major advantages of the RNAi technology over other nucleic acid-based gene targeting approaches. Some examples of applications of RNAi in oncology are given in the following text.

12.3.1.1 Allele-Specific Inhibition

A novel and theoretically even better way to attack cancer cells specifically is not to attack cancer cells based on their genetic gains compared with normal cells, but rather through vulnerabilities created by genetic losses. Many types of cancer cells lose genetic sequences irreversibly during tumorigenesis, which may be exploited

as a vital weak point in their defense. The loss of large chromosomal regions, or even whole chromosomes, is an early event in the clonal evolution of cancers. The so-called loss of heterozygosity (LOH) is a consequence of mitotic nondisjunction during the process of tumorigenesis. The vulnerabilities created via LOH are one of the few (if not only) widely occurring, absolute, and consistent weak spots exhibited by tumor cells.

Allele-specific inhibition (ASI) is an approach where cancer cells are attacked at LOH. The rationale for ASI therapy is based on the fact that regions involved in LOH are thought to contain tumor suppressor genes, and LOH can involve 20 % of the total genome in certain cancers. Therefore, the genetic difference between normal cells and cancer cells extends beyond the loss of a tumor suppressor gene. ASI is based on the following rationale:

- Tumor cells carry large regions of LOH.
- LOH regions contain genes essential for cell survival.
- These genes show genetic variation in their mRNA-coding sequences.
- Both alleles are expressed in normal tissue.
- Inhibition of the allele retained in the tumor is cytotoxic and can be achieved by ASI.

siRNA possesses unique characteristics which imply that siRNA cannot only be used as a tool to study gene function, but might also be used as genotype-specific drug to mediate ASI. First, like antisense oligonucleotides, siRNA is highly sequence specific. Single-nucleotide mismatches abrogate the ability to suppress gene expression. It is possible to specifically inhibit the mutant K-ras allele using retroviral delivery of siRNA, while leaving the wild-type K-ras allele untouched. Therefore, ASI is possible with siRNA. With our current knowledge it seems that the greater versatility of siRNA design as compared might allow for targeting more SNPs within an essential gene suitable for ASI than is possible with antisense oligonucleotides. ASI has been demonstrated in tumors using a retroviral-mediated siRNA delivery system. These results suggest that the prospects for implementing ASI through siRNA are quite promising.

12.3.2 Inhibition of Oncogenes

Oncogenes found in human cancer often differ by only single base mutations from their normal (i.e., wild-type) counterparts—genes that may be required for viability of normal cells. No technology is available to date to specifically inactivate only the mutant versions of such oncogenes. This may be essential for safe and effective anticancer therapy, as the corresponding wild-type alleles may be required for viability of normal cells. Using the pSUPER.retro vector, successful retroviral delivery of siRNAs has been reported at the Netherlands Cancer Institute, which leaves the wild-type K-ras allele untouched while specifically inhibiting the mutant K-RASV12 allele in human pancreatic carcinoma resulting in loss of tumorigenicity and oncogenic phenotype. This was the first evidence in living animals that

cancer can be controlled by blocking the expression of a single mutant protein using the technique of RNAi.

Human papilloma virus (HPV) plays an important role in causing cervical cancer. Targeting of HPV oncogenes requires efficient delivery of siRNA. Specific knockdown of the HPV18 E6 and E7 oncogenes has been achieved using the X-tremeGENE (Roche) siRNA reagent with resulting efficient inhibition of the growth of HPV-positive cervical cancer cells (Wise-Draper and Wells 2005).

CEQ508 (Marina Biotech Inc.), an orally administered tkRNAi drug candidate, targets beta-catenin, a key oncogene implicated in the formation of colonic polyps and in the progression of polyps to colorectal cancer. It is in a phase Ib/IIa trial for familial adenomatous polyposis patients.

Cancer-associated genes that can be targeted by RNAi are listed in Table 12.3.

12.3.3 Modification of Alternative Splicing in Cancer

The bcl-X gene is an example of how alternative splicing affects the course of cancer. If the gene is spliced to include all of exon 2, it will produce bcl-XL mRNA, which results in a protein that inhibits apoptosis. If the gene is spliced without a portion of exon 2, it produces bcl-XS mRNA, which in turn creates an apoptosis-inducing protein. Many cancers have a high incidence of bcl-XL, while successful chemotherapy results in a higher proportion of bcl-XS. Splicing can be modified by antisense therapy as well as RNAi.

RNAi approach has been tested in SNB19 glioblastoma cells where SR (serine-/arginine-rich) p55 plays a major role in maintaining normal FGFR (fibroblast growth factor receptor) 1 alpha-exon inclusion. FGFR1 gene transcript is alternatively processed to produce functionally different receptor forms. An RNAi-mediated approach led to 6- to 14-fold decrease in exon inclusion on ablation of SRp55 (Jin and Cote 2004). These observations indicate that SRp55 plays a major role in maintaining normal FGFR1 alpha-exon inclusion, which is subject to dominant intronic splicing silencer-mediated and exonic splicing silencer-mediated inhibition in SNB19 cells.

12.3.4 Onconase

Onconase (ranpirnase) is one of the several cytotoxic ribonucleases (CRs), homologs of the pancreatic RNase A, have been isolated from amphibian oocytes or embryos. It shows antitumor properties and is in phase III clinical trials by Alfacell Corporation. Ranpirnase is a promising candidate as an enhancer for radiation therapy and chemotherapy (Lee 2008). Owing to its selective destruction of malignant cells and favorable toxicology profile, ranpirnase is a promising antitumor agent with ideal attributes that are generally lacking in conventional cytotoxic drugs

Table 12.3 Cancer-associated genes that can be targeted by RNAi

Gene target	Mechanism	Comments
53BP1	DNA damage checkpoint protein	siRNA was used to demonstrate this mechanism
BAG-1	Apoptosis	Human cervical cancer HeLa cells with downregulated BAG-1 levels can be obtained by using RNAi
Bcr–Abl	Oncoprotein	RNAi can inhibit oncogenes
C-raf and bcl-2	Chemoresistance and apoptosis	Combined RNAi of c-raf and bcl-2 genes may represent a novel approach to leukemia
CEACAM6	An immunoglobulin implicated in apoptosis and overexpressed in cancer	CEACAM6 warrants further investigation as a novel therapeutic target for the treatment of pancreatic adenocarcinoma
CypA (cyclophilin A)	Overexpressed in non-small-cell lung cancer (NSCLC)	RNAi-mediated knockdown slows tumor growth and increases apoptosis; this may lead to targeted therapies for NSCLC
DEAD box RNA helicase	Transcriptional regulation	RNA helicases can associate with nuclear receptors and function as coregulators to modulate receptor transcriptional activity
DIP (DDC interacting protein) 13α	Tumor suppressor	DIP13α expression by siRNA blocks DDC (deleted in colorectal cancer)-induced apoptosis
DNMT1	Hypermethylation	DNMT1 is required to maintain global methylation and aberrant CpG island methylation in human cancer cells
ErbB1	Oncoprotein	siRNA-mediated inhibition of erbB1 is a useful therapeutic approach in treatment of cancer
FGF-4	Oncoprotein	FGF-4 siRNA complex inhibits tumor growth
FLIP (FLICE-inhibitory protein)	Apoptosis	Inhibition of FLIP expression is sufficient to sensitize tumor cells for TRAIL-induced apoptosis
Human papillomavirus type 16 E5 and E7	Viral oncoprotein	siRNA can induce selective silencing of exogenous viral genes
Huntingtin Interacting Protein 1 (HIP 1)	Oncoprotein for prostate cancer	Transcription is suppressed when knocked down by RNAi approach
K-ras	Oncoprotein	Cancer can be controlled by blocking the expression K-ras using of RNAi
KITENIN	Promotes invasion of mouse colon adenocarcinoma cells in vivo	In vivo KITENIN ablation using pSUPER-KITENIN vectors producing siRNA can function as a chemotherapeutic against colon cancer
MBD2	Hypermethylation	With siRNA targeting of MBD2 mRNA, there is no repression of luciferase reporter expression
MCL1/fortilin	Apoptosis	MCL1, an antiapoptotic molecule, binds and stabilizes fortilin in vivo
MDR1	Multiple drug resistance	RNAi can modulate MDR in preclinical models
$P21^{Cip1/Waf1}$	Cell cycle dysregulation	Suppression of p21 by siRNA
Skp-2	Dysregulation of cell cycles in various cancers	Adenovirus vector-mediated siRNA for Skp-2 inhibits tumor growth
VEGF	Angiogenesis	Vector-based RNAi for knockdown of VEGF and antiangiogenesis gene therapy of cancer

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(Beck et al. 2008). Degradation of tRNA by Onconase internalized into cells that leads to inhibition of protein synthesis is considered the mechanism of its cytotoxicity. Several findings, however, cannot be explained by nonspecific decline in protein synthesis alone and suggest additional or alternative mechanism(s).

A study reported that the silencing of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene in human lung adenocarcinoma A549 cells by siRNA was effectively prevented by Onconase (Zhao et al. 2008). Although transfection of cells with GAPDH siRNA reduced expression of this protein by nearly 70 %, the expression was restored in the cells exposed to Onconase for 48 or 72 h. The data thus provide evidence that one of the targets of Onc is siRNA, likely within RISC. In light of the findings that miRNAs are involved in tumor pathogenesis as well as in enhancing cell resistance to anticancer therapy, these data may provide explanation for both the antitumor Onconase activity and its propensity to enhance effectiveness of cytotoxic drugs.

12.3.5 Overcoming Drug Resistance in Cancer

Resistance to chemotherapeutic drugs is a major problem in cancer treatment. P-glycoprotein encoded by MDR1 is a multidrug transporter with a major role in MDR. MDR1-targeted RNAi can decrease MDR1 mRNA, abolish P-glycoprotein expression, and completely reverse the MDR phenotype. These target sequences can be integrated into gene therapy vectors with potential clinical application. MDR can be reversed by the shRNA-mediated MDRI suppression in human hepatoma cell lines, which provides a valuable clue to make multidrug-resistant hepatoma cells sensitive to anticancer drugs (Chen et al. 2006).

Engineered pieces of protein-encoding RNA have been used to target repair proteins in cancer cells, effectively shutting the RNA down. Such a "gene silencer" can block a cancer cell's ability to repair itself after damage due to drugs and radiation. Unable to make the necessary repair proteins, cancer cells then become susceptible to chemotherapy and radiation treatments. Some siRNAs may be able to silence genes by methylating their promoters. By exploiting this pathway, mRNA levels of an oncogene, erbB2, can be reduced by more than 80 % in malignant human mammary cells, significantly reducing their proliferation (Kawasaki and Taira 2004). Thus, siRNAs might have potential as a new type of gene therapeutic agents.

12.3.6 RNAi-Based Logic Circuit for Identification of Specific Cancer Cells

An RNAi-based synthetic circuit, known as a cell-type classifier, has been developed to distinguish between cancerous and noncancerous cells. It senses expression levels of a customizable set of 5 endogenous miRNAs within a population, in order

to selectively identify HeLa cells and trigger their apoptosis without affecting non-HeLa cell types (Xie et al. 2011). The overall network was designed to implement a multi-input, AND-like logic function for identification and selective killing of HeLa cells through regulated expression of hBax. The potential therapeutic applications of classifier circuits obviously require efficient in vivo DNA delivery to cells. Besides treatment of cancer, the classifiers could also be used for in vitro applications such as drug screening or monitoring developmental processes.

12.3.7 shRNA-Based Autologous Cancer Vaccine

The FANG vaccine (Gradalis Inc.) is an autologous tumor-based product incorporating a plasmid expressing a well-established immune activator, granulocyte–macrophage colony-stimulating factor (GM-CSF), and a novel bifunctional shRNAi (bi-shRNAi) targeting furin convertase, thereby downregulating endogenous immunosuppressive TGF-β1 and TGF-β2. It is manufactured from a cell suspension derived from a portion of a patient's tumor removed during surgery. bi-shRNAi is introduced into the cells by electroporation. Cells are then incubated overnight, irradiated, frozen, tested, and released. Vaccine is shipped to the patient's clinic where doses are thawed and administered monthly by intradermal injection. FANG manufacturing is a straightforward 2-day cGMP process that is applicable to nearly all tumor types with no modification, and it does not require patients to undergo apheresis or other treatments except surgical tumor removal if indicated.

Results of a phase I study showed that treatment with FANG was safe and significantly increased survival in patients with advanced stage cancer compared to patients who received other forms of treatment (Senzer et al. 2012). The vaccine elicits a robust and lasting immune response, resulting in statistically significant prolonged survival in patients with advanced stage disease. Currently, FANG is being evaluated in several phase II trials in patients with advanced ovarian cancer, advanced melanoma, and advanced colorectal cancer with liver metastases. In addition, Gradalis has initiated a clinical program evaluating FANG in children with Ewing's sarcoma.

12.3.8 siRNAs for Inducing Cancer Immunity

Cancer vaccination aims at stimulating a systemic immune response targeted to antigens expressed in the disseminated tumor lesions. Expression of potent antigens can be induced in tumor cells by siRNA-mediated inhibition of nonsense-mediated messenger RNA decay (NMD) leading to the expression of new antigenic determinants and their immune-mediated rejection (Pastor et al. 2010). Preventing this process of NMD enables the cells to produce aberrant proteins in situ, which are viewed as foreign and attacked by the immune system.

In subcutaneous and metastatic tumor models, tumor-targeted delivery of NMD factor-specific siRNAs conjugated to oligonucleotide aptamer ligands led to significant inhibition of tumor growth that was superior to that of vaccination with GM-CSF-expressing irradiated tumor cells and could be further enhanced by costimulation. An aptamer linked to an siRNA, designed to inhibit the NMD process, specifically targeted PSMA expressed on prostate cancer cells and eliminated tumors in cancer-bearing mice.

Tumor-targeted NMD inhibition forms the basis of a simple, broadly useful, and clinically feasible approach to enhance the antigenicity of disseminated tumors leading to their immune recognition and rejection. The cell-free chemically synthesized oligonucleotide backbone of aptamer–siRNAs reduces the risk of immunogenicity and enhances the feasibility of generating reagents suitable for clinical use. The NMD inhibition strategy is simple, consisting of a single reagent that can be synthesized by a cell-free chemical process and obviates the need for adjuvants. The cell-free, chemically synthesized oligonucleotide backbone of aptamer–siRNAs reduces the risk of immunogenicity. It is broadly applicable as it targets a pathway common to all cancers.

12.3.9 siRNAs for Inhibition of Angiogenesis

Researchers at Intradigm Corporation are using an siRNA targeting system to modulate the rate of tumor growth and to determine which genes are likely to correlate with therapeutic efficacy. They have previously established that by using certain agents, including lipids and polymers, to carry siRNAs into cells, they could minimize the "biological noise" that hampers plasmid and viral DNA delivery methods. siRNAs, when injected into tumors of living mice, get into cancer cells and modulate not only expression of reporter genes, such as beta-galactosidase, but also the RNAs that are relatively evenly distributed throughout the tumor. siRNAs against the gene for VEGF into a mouse mammary tumor led to a decrease in the tumor growth rate and a decrease in the microvasculature that supports such growth. The VEGF RNA and protein levels are both reduced by about 50 % suggesting that the decreased growth rate is due to VEGF-specific responses to the siRNA and not to secondary effects. This suggests that siRNAs inhibited angiogenic pathways in the tumor with, which slowed their growth. The Intradigm team then used microarray analysis to compare gene expression levels in these tumors with those in controls. They identified approximately 300 previously uncharacterized genes whose expression levels were altered in response to inhibition of angiogenesis. These genes potentially correlate with a positive therapeutic outcome because their expression correlates with slowed growth.

To retest these potentially interesting genes, the researchers synthesize siRNAs that match their sequence and then inject them into the tumors to see what effect

they have on the tumor growth rate. Thus far, the team has retested 50 of the genes discovered in the initial screen and has identified six that correlate with slower growth. Two of these genes are involved in apoptosis. It will soon be possible to test such intratumoral siRNA injections as potential therapeutics for certain human cancers, including head and neck cancers and melanoma.

12.4 Clinical Trials of RNAi-Based Therapies for Cancer

A number of RNAi-based products are now in clinical trials. Table 12.4 shows clinical trial status of RNAi-based or related therapeutics for cancer. Sponsors include mostly companies but some academic centers as well.

Table 12.4 Clinical trials of RNAi-based therapeutics

Company/ Institution	Product/target	Indication	Clinical trial status
Alnylam	ALN-VSP: siRNA formulated with LNP (Tekmira Pharmaceuticals)	Liver cancer	Phase I
Calando Pharmaceuticals	CALAA01, a targeted siRNA, employs a novel nanoparticle delivery system Calando's RONDEL™	Cancer	Phase Ib
Duke University (Durham, NC)	Proteasome siRNA and tumor antigen RNA-transfected dendritic cells	Metastatic melanoma	Phase I
Gradalis	Bifunctional shRNA technology with an immune system stimulator	Cancer	Phase I
Marina Biotech Inc.	CEQ508, an orally administered tkRNAi/beta-catenin, an oncogene implicated in colorectal cancer	Familial adenomatous polyposis	Phase Ia/IIb
MD Anderson Cancer Center	EphA2 gene targeting using siRNA-EphA2-DOPC intravenous delivery	Advanced solid cancers	Phase I
Merck/Sirna Therapeutics	AGN-745 (Sirna-027) chemically modified siRNA targeting VEGF R-1	Age-related macular degeneration	Phase II completed
National Cancer Institute (NCI)	Hepatic intra-arterial administration of TKM 080301 (lipid nanoparticles containing siRNA against PLK1 gene)	Cancers with hepatic metastases	Phase I completed
Silence Therapeutics	Atu-027; siRNA targeting the protein kinase PKN-3	Solid tumors	Phase I completed
Silenseed	siG12D LODER (Local Drug EluteR)	Pancreatic cancer	Phase I
Tekmira Pharmaceuticals	TKM-PLK1: LNP formulation of siRNA targeting polo-like kinase 1	Cancer	Phase I
TransDerm Inc.	TD101 targets disease mutation	Pachyonychia congenita	Phase Ib

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12.4.1 Targeted Delivery of a Nanoparticle–siRNA Complex in Cancer Patients

The first in-human phase I clinical trial involving the systemic administration of siRNA to patients with solid cancers using a targeted, nanoparticle delivery system (Calando's CALAA01 and the RONDEL™ delivery system) is in progress (Davis et al. 2010). Nanoparticles had an attached ligand for a receptor that is more abundant on cancer cells to ensure that they were taken up predominantly by those cells. Nanoparticles, measuring ~70 nm in diameter, are administered intravenously to patients, where they circulate until they encounter "leaky" blood vessels that supply the tumors. The nanoparticles then pass through the vessels to the tumor, where they bind to the cells and are then absorbed. Once inside the cell, the nanoparticles disintegrate and release the siRNA, which evades the immune system and is delivered to the tumor cells. The dissolved parts of the nanoparticle are <50 nm in diameter and are excreted in urine. There is evidence of induction of RNAi mechanism of action in a human from the delivered siRNA. Tumor biopsies from melanoma patients obtained after treatment show the presence of intracellularly localized nanoparticles in amounts that correlate with dose levels of the nanoparticles administered. Furthermore, a reduction was found in both the specific mRNA—RRM2—and protein levels as compared to pre-dosing tissue. The presence of an mRNA fragment demonstrates that siRNA-mediated mRNA cleavage occurs specifically at the site predicted for an RNAi mechanism from a patient who received the highest dose of the nanoparticles. The nanoparticles had inhibited expression of the key gene, RRM2, needed for the cancer cells to multiply. These data demonstrate that siRNA administered systemically to a human can produce a specific gene inhibition (reduction in mRNA and protein) by an RNAi mechanism of action. There is a concern that the treatment has not been tested on more patients and that more samples were not taken from each patient. The lower level of protein was found in only one patient. More data is needed from clinical trials to ensure that such therapies are safe for use in humans, but this study provides direct evidence that nanoparticle-mediated RNAi can be used to knock down genes in cancer patients and reduce production of harmful proteins. Improvement due to immune activation is ruled out as the nanoparticle–siRNA complex evaded the immune system. Since the genes that are targeted for knockdown by siRNA are fairly ubiquitous across cancer types, this therapy has the potential to work in other types of cancer. However, some cancers are less dependent on this particular gene for survival and may not be as susceptible to the genetic knockdown as others. Eventually, specific targeting ligands will need to be developed for specific genes. It remains to be seen if the new therapy improves outcome of patients.

12.4.2 Improving Efficacy of siRNAs for Clinical Trials by Improved Delivery

One strategy for clinical development of siRNAs is to improve their efficacy by mixing RNA with compounds that can facilitate uptake by cells and tissues that

normally resist entry of siRNAs. These agents, which include peptides, nanoparticles, and lipids, can also increase protection of the RNA against nucleases by interacting with the RNA surface and restricting access to enzymes in the serum. Several studies have reported gene silencing in animals using complex formulations that contain siRNA. However, these formulations require introduction of an additional chemical agent into patients, complicating the procedure and increasing the potential for unexpected toxicities. The benefit of formulations therefore needs to be substantial to justify the added complexity of the approach. The second is that formulations may lessen the need for chemical modifications because the RNA complex might be more protected from nucleases and more likely to enter the target tissue. However, as with local delivery, it is still probable that chemical modifications will play a role in identifying optimal agents for clinical trials.

12.5 Advantages of RNAi in Oncology

Benefits of RNAi-based therapies are as follows:

- RNAi can be rationally designed to block the expression of any target gene, including genes for which traditional small-molecule inhibitors cannot be found.
- High specificity as RNAi-based therapies specifically inhibits production of single proteins.
- RNAi can persist longer than traditional drugs in cells, leading to biweekly or weekly dosing regimens.
- Due to their sequence specificity, siRNAs have dramatically improved toxicity profiles compared to conventional anticancer agents.
- Relative ease of synthesis and low cost of production (compared to proteins such as antibodies or recombinant growth factors at the concentration needed for therapeutic effects) make siRNAs an attractive new class of small-molecule drugs.
- siRNAs are chemically stable and can be stored lyophilized without refrigeration.
- RNAi is usually reversible. If a nonreversible effect is the goal, genes that encode double-stranded RNA can be inserted into cells so that heritable gene silence is achieved, making RNAi essentially a form of gene therapy.

12.6 Limitations and Drawbacks of RNAi

The race by companies to design siRNA sequences to every gene is of some concern because some of the companies are not taking into account all of the possible variables in their siRNA design, which can cause nonspecific effects. Off-target effects are another concern. One limitation of RNAi is that gene expression is not completely eliminated and the effectiveness of different siRNAs varies. There is difficulty in applying this strategy to cells from higher organisms, such as mammals, because the presence of another dsRNA pathway that activates the interferon pathway.

This high specificity also implies that the application of RNAi in some instances, such as to treat cancer, might lead to resistance due to sequence mutations. However, unlike resistance to other small molecules, which leads to an expensive and time-consuming search for new therapeutic agents, resistance to RNAi may be overcome by introducing a new siRNA that targets a different site on the same mRNA. Moreover, siRNAs that target conserved sequences or multiple sequences at once may provide a way around this problem.

Side effects can result from unintended interaction between an siRNA compound and an unrelated host gene. If RNAi compounds are designed poorly, there is an increased chance for nonspecific interaction with host genes that may cause adverse effects in the host. Interferon response is the best known adverse effect of siRNAs. Although shRNAs can provide stable gene silencing via RNAi, recent studies have shown toxicity in vivo that appears to be related to saturation of the endogenous miRNA pathway.

12.7 Concluding Remarks and Future Prospects of RNAi in Oncology

RNAi has made remarkable progress and impact on biological research and the pharmaceutical industry has already become obvious. Direct introduction into development of therapeutics is being approached with caution. Because it exploits a preexisting mechanism, RNAi is several times more potent than either of the other two types of RNA strategies—antisense and ribozymes.

12.7.1 Challenges for the Development of RNAi-Based Therapeutics

The most significant opportunities and challenges of RNAi-based therapeutics over the coming years will be the successful application of gene silencing for treatment of cancer. Other technological advances that are needed are:

- Ability to target the siRNA to specific organs or tissues
- Chemical modifications that enhance siRNA stability
- Chemical modifications that improve the specificity of target gene knockdown
- Chemical modifications that improve the bioavailability and pharmacokinetics
- Expression-based delivery methods that limit the risks of adverse genomic insertion events and allow careful control of expression levels
- Reliable and low-cost methods for the large-scale production of siRNA sufficient to support clinical evaluation and therapeutic deployment

Off-target effects of siRNAs and adverse effects of RNAi need attention. It is clear now that RNAi machinery tolerates some sequence mismatches, i.e., genes can be silenced by siRNA that are only partially complementary to their sequence. The level of mismatch is still under investigation. Because the mechanisms of off-target effects are only partially understood, the only remedy at this stage is the use of specific controls.

One of the big hurdles is delivery of siRNAs. Even if delivery problems can be solved, other questions remain, including that of whether therapeutic levels of RNAi may be toxic. In case of cancer, the target of interest may be in normal cells as well as cancer cells. The problem can be solved by increasing specificity but it is unclear whether highly specific drugs produce a high therapeutic effect. Most active anti-cancer drugs have multiple mechanisms of action. If they are made more specific, the therapeutic activity is likely to be reduced. Difficulties encountered with antisense and gene therapy should caution that the likely problems with RNAi should not be underestimated, but it does not mean that they cannot be overcome.

RNAi is still considered to be an immature technology. However, the current progress indicates that RNAi has better prospects than antisense oligonucleotides. Despite the questions and unsolved problems, several companies are moving ahead to develop RNAi therapy. The outstanding questions are probably only likely to be answered in the process of clinical trials, many of which are in progress. The first applications are likely to be in cancer (targeting oncogenes). To avoid some of the problems of delivery, initial trials may deliver the siRNA by direct injection into the target tissue (for a tumor, for instance) or ex vivo, treating white blood cells infected with HIV.

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Chapter 13
Role of MicroRNAs in Cancer

13.1 Introduction

MicroRNAs (miRNAs), small and mostly noncoding RNA gene products, are molecules derived from larger segments of "precursor" RNA that are found in all diverse multicellular organisms. miRNAs are 21–25 nucleotide transcripts that repress gene function through interactions with target mRNAs. More detailed information is contained in a special report on microRNAs (Jain 2014).

The primary transcripts of miRNAs contain dsRNA and are therefore potential substrates for adenosine to inosine (A-to-I) RNA editing. RNA editing increases the diversity of miRNAs and their targets and hence may modulate miRNA function. miRNAs target the control of gene activity at multiple levels, specifically transcription, translation, and protein degradation, i.e., miRNAs act as metaregulators of expression control. A schematic miRNA pathway is shown in Fig. 13.1.

The hairpin, known as precursor miRNA (pre-miRNA), has a two-nucleotide overhang at its 3′ end; it has 3′ hydroxyl and 5′ phosphate groups. It is cleaved by the RNase III enzyme Dicer, which interacts with the 3′ end of the hairpin and cuts away the loop joining the 3′ and 5′ arms, yielding an imperfect miRNA–miRNA duplex about 22 nucleotides in length. Although either strand of the duplex may potentially act as a functional miRNA, only one strand is usually incorporated into the RNA-induced silencing complex (RISC) where the miRNA and its mRNA target interact.

Each miRNA is thought to regulate multiple genes, and since numerous miRNA genes have been identified in humans, the potential regulatory circuitry afforded by miRNA is enormous. Thousands of miRNA entries have been registered and miRNAs have been implicated in most, if not all, central cellular processes and several diseases. It is estimated that up to 30 % of human genes may be miRNA targets. Some miRNAs have been reported to regulate the expression of genes involved in differentiation and cell growth, tumor suppressors and oncogenes.

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_13,
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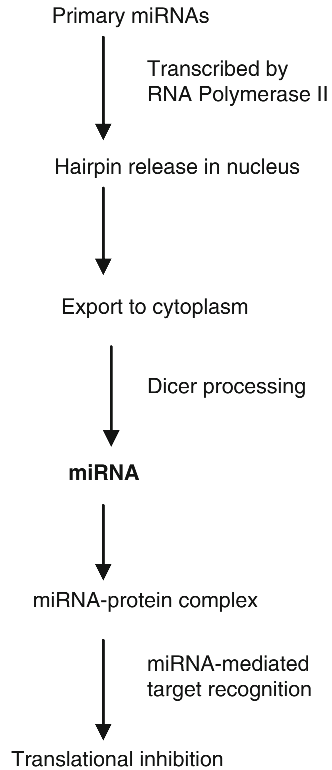

Fig. 13.1 A schematic miRNA pathway

13.2 miRNAs Compared to siRNAs

miRNAs are derived by processing of shRNAs that can inhibit the translation of mRNAs bearing partially complementary target sequences. miRNAs are the naturally occurring counterparts of siRNAs. Like siRNAs, miRNAs are involved in the RNAi mechanism. miRNAs may function via a similar enzyme complex as siRNAs. However, while siRNAs direct the cleavage of mRNA synthesized by a gene, miRNAs appear to predominantly block translation of proteins by binding to the mRNA.

In contrast, siRNAs, which are derived by processing of long dsRNAs and are often of exogenous origin, degrade mRNAs bearing fully complementary sequences. An endogenously encoded human miRNA is able to cleave an mRNA bearing fully complementary target sites, whereas an exogenously supplied siRNA can inhibit the expression of an mRNA bearing partially complementary sequences without inducing detectable RNA cleavage. Thus miRNAs and siRNAs can use similar mechanisms to repress mRNA expression and the choice of mechanism may be largely or entirely determined by the degree of complementarity of the RNA target. Despite similar mechanisms, siRNAs might still be more effective at RNA degradation than

at translation inhibition, whereas miRNAs might display the converse activity. An siRNA will direct translational repression of a target site with partial complementarity, whereas an miRNA can direct cleavage of a fully complementary target mRNA. As miRNA and mRNA have to be present simultaneously at minimum levels in the same cellular compartment for a biologically meaningful interaction, more precise expression data as a function, e.g., of developmental stage, will be extremely useful. Transfecting cells with siRNAs rather than larger dsRNAs avoids the nonspecific gene silencing of the interferon response, underscoring the importance of developing efficient methods for producing reliable siRNAs. The mechanisms by which siRNAs and miRNAs induce gene silencing are complementary to each another, thereby enabling a dual approach to harnessing the RNAi mechanism to downregulate pathogenic proteins and viruses.

13.3 Role of miRNAs in Cancer

Loss or amplification of miRNA genes has been reported in a variety of cancers, and altered patterns of miRNA expression may affect cell cycle and survival programs. Germ line and somatic mutations in miRNAs or polymorphisms in the mRNAs targeted by miRNAs may also contribute to cancer predisposition and progression. Onco-miRNAs (also called oncomirs) and suppressor miRNAs can represent two different looks of the same gene like Dr Jekyll and Mr Hyde, behaving as oncogenes or tumor suppressors depending on tissue type and specific targets (Fabbri et al. 2007). Alterations in miRNA genes play a critical role in the pathophysiology of many, perhaps all, human cancers.

13.3.1 miRNA Genes

One study found that 2.5 % of miRNA genes are in cancer-associated genomic regions or in fragile sites and Northern blotting showed that several miRNAs located in deleted regions had low levels of expression in cancer samples (Calin et al. 2004). These data provide a catalog of miRNA genes that may have roles in cancer and indicate that the full complement of miRNAs in a genome may be extensively involved in cancers.

Copy number alterations of miRNAs and their regulatory genes are highly prevalent in cancer and may account partly for the frequent miRNA gene deregulation reported in several tumor types. miRNAs can behave like oncogenes, which promote tumor growth, or tumor suppressors, which keep potentially malignant cells in check. miRNA activity is tissue-sensitive, meaning some miRNAs may be overexpressed, or “turned on,” in some of the cancers while in others they are underexpressed, or “turned off.” Increased expression of miR-21, an oncogene, leads to

increased human telomerase reverse transcriptase (hTERT) expression and increased telomerase activity causes cell immortalization—characteristic of cancer. Decreased expression of let-7, a tumor suppressor, increases expression of oncogene RAS and induces cell proliferation—another feature of cancer (Pillai et al. 2005).

miRNA genes are frequently located in cancer-associated regions of the genome, e.g., at fragile sites, as well as in minimal regions of loss of heterozygosity, minimal regions of amplification (minimal amplicons), or common breakpoint regions. The regions of the genome that are consistently involved in chromosomal rearrangements in cancer cells but that lack oncogenes or tumor suppressor genes appear to harbor miRNA genes, which can act both as tumor suppressor genes and oncogenes. The classic paradigm in oncogenesis is the accumulation of mutations in the open reading frames of protein-encoding oncogenes and tumor suppressors. miRNA genes, unlike other genes involved in cancer, do not encode proteins. The identification of miRNAs that are important for development and cell homeostasis will likely change this current paradigm of cancer.

Genomic alteration of miRNA genes may constitute a critical step in cancer development because they are highly frequent in epithelial cancers and result in changes in mature miRNA expression (Zhang et al. 2006). Genetic alterations of miRNAs may thus promote and/or enhance alteration of protein-encoding gene expression in cancer, accelerating malignant transformation and/or tumor growth. These results may contribute to a better understanding of the pathogenesis and the identification of biomarkers and targets for human cancer. Targeting of miRNAs may provide an important therapeutic strategy for human cancer.

13.3.2 miRNAs Regulation by Posttranscriptional Mechanisms

Posttranscriptional mechanisms regulate miRNA abundance during development as well as in cancer cells where miRNAs frequently exhibit dysregulated expression. Loss or amplification of miRNA genes has been reported in a variety of cancers, and altered patterns of miRNA expression may affect cell cycle and survival programs. Posttranscriptional silencing of target genes by miRNAs occurs either by targeting specific cleavage of homologous mRNAs or by targeting specific inhibition of protein synthesis.

Germ line and somatic mutations in miRNAs or polymorphisms in the mRNAs targeted by miRNAs may also contribute to cancer predisposition and progression. The molecular mechanisms that govern the global efficiency of miRNA biogenesis in these settings remain incompletely understood, and experimental systems for the biochemical dissection of these pathways are currently lacking. One study has demonstrated that miRNAs are subject to dynamic posttranscriptional regulation in widely used cell culture systems (Hwang et al. 2009). As diverse mammalian are grown to increasing density, miRNA biogenesis is globally activated, leading to elevated mature miRNA levels and stronger repression of target constructs. This

broad increase in miRNA abundance is associated with enhanced processing of miRNAs by Drosha, an RNase III endonuclease, and more efficient formation of RNA-induced silencing complexes. These findings uncover a critical parameter necessary for accurate analysis of miRNAs in cell culture settings, establish a tractable system for the study of regulated miRNA biogenesis, and may provide insight into mechanisms that influence miRNA expression in physiologic and pathophysiologic states. A multisubunit protein complex termed "microprocessor" is necessary and sufficient for processing miRNA precursor RNAs. Microprocessor contains Drosha and DGCR8, a gene deleted in DiGeorge syndrome. These findings link miRNA perturbation to cancer.

13.3.3 Oncomirs

The expression profiles of oncomirs can be used for the classification, diagnosis, and prognosis of human malignancies (Deng et al. 2009). miRNA expression in human epithelial cancers may be upregulated or downregulated as shown in Table 13.1.

The patterns of miRNA alterations reported in cancer versus normal tissues are very likely the sum of a large variety of highly complex molecular signals, including activation of oncogenic pathways. However, other well-documented microenvironmental factors, such as pH alterations, local decrease in glucose levels, paracrine growth factors, and tumor–stromal interactions could add to the complexity of miRNA deregulation in cancer. There is a link between hypoxia, a key feature of the tumor microenvironment, and a group of miRNAs. Select members of this group seem to affect apoptotic signaling in a hypoxic environment and are also predicted to target genes of critical importance for tumor biology. Overwhelming majority of hypoxia-related miRNAs are also overexpressed in at least some types of tumor types, suggesting that hypoxia may represent a contributing element for miRNA alterations in cancer (Kulshreshtha et al. 2007).

One cluster of miRNAs, the miR-17-92 polycistron, is located in a region of DNA that is amplified in human B-cell lymphomas and the levels of the primary or mature microRNAs derived from the miR-17-92 locus are often substantially increased in these cancers (He et al. 2005a). Enforced expression of the miR-17-92 cluster acted with c-myc expression to accelerate tumor development in a mouse B-cell lymphoma model. Tumors derived from HSCs expressing a subset of the miR-17-92 cluster and c-myc could be distinguished by an absence of apoptosis that was otherwise prevalent in c-myc-induced lymphomas. In another study, a set of five miRNAs, including the three most upregulated ones (miR-221, miR-222, and miR-146), distinguished unequivocally between papillary thyroid carcinoma and normal thyroid indicating their involvement in the pathogenesis of carcinoma (He et al. 2005b). Another study has shown that marked overexpression of the miR-17-92 cluster with occasional gene amplification may play a role in the development of lung cancers, especially in

Table 13.1 Dysregulation of miRNA expression in epithelial cancers

Cancer	miRNA upregulation	miRNA downregulation
Breast	let-7f, miR-21, miR-22, miR-155, miR-196-2, miR-155, miR-181b, miR-203, miR-205, miR-210, miR-365	let-7, let-7a, miR-31, miR-93, miR-101-1, miR-125a, miR-125b, miR-127d, miR-145, miR-204, miR-205, miR-320, miR-497, miR-355
Cervical	miR-9, miR-15b, miR-16, miR-21, miR-133-a/b, miR-145, miR-146a, miR-155, miR-199-s, miR-199-a/b, miR-127, miR-214	miR-126, miR-143, miR-145, miR-149, miR-203
Colorectal	miR-17-5p, miR-20a, miR-21, miR-31, miR-92, miR-191, miR-155, miR-200c, miR-226	let-7, let-7a, miR-106a, miR-143, miR-145, miR-148b, miR-192, miR-215, miR-320, miR-331, miR-342, miR-498
Gastric	miR-21, miR-191, miR-215, miR-223	let-7 family, miR-138-2, miR-152-prec
Liver	miR-18, miR-18-prec, miR-221, miR-224	miR-125a, miR-195, miR-199a, miR-200a
Lung	miR-17–92, miR-21, miR-137, miR-191, miR-210, miR-155, miR-182, miR-205, miR-372	let-7, 7a, let-7a2, miR-126, miR-143, miR-192-prec, miR-221, miR-224
Ovarian	miR-29a, miR-99a, miR-199a/b, miR-200a/b/c, miR-141, miR-214, miR-221, miR-296, miR-302d, miR-424, miR-494	let-7 family, miR-100, miR-106b, miR-122a, miR-125b1, miR-141, miR-183, miR-195, miR-199a, miR-140, miR-145, miR-200a, miR-335, miR-424
Pancreatic	miR-21, miR-103, miR-107, miR-155, miR-196a-2, miR-210, miR- 213, miR-221, miR-181a	miR-148a, miR-148b, miR-375
Prostate	let-7d, miR-17-5p, miR-21, miR-26a, miR-29b-2, miR-31, miR-32, miR-99a, miR-182, miR-191, miR-195, miR-200c, miR-203, miR-210, miR-223, miR-296, miR-320, miR-370, miR-503	let-7 family, let-7b, miR-9-3, miR-15a, miR-16, miR-16-1, miR-24, miR-26, miR-27a, miR-29, miR-30, miR-34a, miR-99, miR-125, miR-128a, miR-129, miR-143, miR-145, miR-161a, miR-200, miR-205, miR-221, miR-222, miR-224, miR-487

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their most aggressive form, small-cell lung cancer (Hayashita et al. 2005). Together, these studies indicated that miRNAs can modulate tumor formation and implicate the miR-17-92 cluster as a potential human oncogene.

13.3.4 Linking miRNA Sequences to Cancer Using RNA Samples

NCode™ (Life Technologies) Human miRNA microarray V3 consists of miRNA content from multiple sources, including the Sanger 10.0 miRNA database and novel miRNAs unavailable in public databases. Use of NCode™ microarray on samples from BioServe's RNAs isolated from fresh-frozen, fully annotated tumors has identified ncRNAs that are differentially expressed in healthy and diseased

tissue. NCode™ Profiler software identified miRNAs that were either up- or down-regulated in tumor versus healthy tissue, and quantitative PCR was used to validate the findings. Novel miRNA sequences thus identified could potentially be involved in the generation of new tumor tissues, particularly in colorectal cancer.

13.3.5 Role of miRNAs in Viral Oncogenesis

Role of miRNAs in viral oncogenesis is not well understood. There is a need for coordinated efforts from multiple laboratories to build a holistic view of host–virus interactions. This would include prediction and validation of genome-scale protein–protein and miRNA–target interactions, along with temporal analysis of gene expression, which could be integrated onto a bioinformatics platform to understand the dynamics and intricacies of host–virus cross talks. Availability of high-throughput expression and proteomics coupled to high-performance operating platforms could enable one to integrate questions and answers in a systems biology manner (Scaria and Jadhav 2007). This collective approach could greatly aid in understanding host–virus interactions in an inclusive way.

13.3.6 miRNAs Interaction with p53

P53 plays a central role in tumor prevention. As a transcription factor, p53 mainly exerts its function through transcription regulation of its target genes to initiate various cellular responses. To maintain its proper function, p53 is tightly regulated by a wide variety of regulators in cells. Various studies have shown that miRNAs interact with p53 and its network at multiple levels (Feng et al. 2011). p53 regulates the transcription expression and the maturation of a group of miRNAs. On the other hand, miRNAs can regulate the activity and function of p53 through direct repression of p53 or its regulators in cells. These findings have demonstrated that miRNAs are important components in the p53 network and also add another layer of complexity to the p53 network.

13.3.7 miRNAs, Embryonic Stem Cells, and Cancer

Posttranscriptional control of miRNA biogenesis has been observed in embryonic stem cells (ESCs), embryonal carcinoma (EC) cells, and primary tumors. These data support the notion that unidentified mechanisms exist in stem cells and certain cancers to prevent miRNA-mediated cell differentiation. Developmentally regulated RNA-binding protein Lin28 has been identified as a selective inhibitor of the maturation of let-7 family miRNAs in embryonic cells. Studies have been carried out to understand the mechanisms by which Lin28 controls miRNA biogenesis, as

well as to identify novel miRNA regulatory pathways. Lin28 proteins act mainly in the cytoplasm by inducing uridylation of precursor let-7 at its 3′ end. The uridylated pre-let-7 (up-let-7) fails Dicer processing and undergoes degradation. The Lin28-mediated downregulation of let-7 may play a key role in development, ESC programming, and tumorigenesis (Heo et al. 2008). Another study has shown that Lin28 blocks processing of let-7 family miRNAs in ESCs by selectively binding to the terminal loop region of let-7 precursors in vitro, which mediates miRNA processing inhibition in vivo (Piskounova et al. 2008). These findings establish a regulatory role for the terminal loop of precursors in miRNA maturation and provide insight into the mechanism by which Lin28 negatively regulates let-7 processing. Knockdown of Dicer dramatically attenuates cell division in human hESCs. Use of microarrays and qPCR analysis has enabled the identification of an hESC-enriched miRNA group (Bar et al. 2008). miRNAs have been identified that can partially rescue the cell division defects observed in hESC Dicer knockdown lines.

13.3.8 miRNAs and Cancer Metastases

Several studies have linked various miRNAs to metastasis that either promote or suppress the malignant process. At the same time, epigenetic silencing of miRNAs with tumor suppressor features by CpG island hypermethylation is also being recognized as a common hallmark of human cancers. For the investigation of an miRNA hypermethylation profile characteristic of human metastasis, a pharmacological and genomic approach has been used to reveal this aberrant epigenetic silencing program by treating lymph node metastatic cancer cells with a DNA demethylating agent followed by hybridization to an expression microarray (Lujambio et al. 2008). Among the miRNAs that were reactivated upon drug treatment, miR-148a, miR-34b/c, and miR-9 were found to undergo specific hypermethylation-associated silencing in cancer cells compared with normal tissues. The reintroduction of miR-148a and miR-34b/c in cancer cells with epigenetic inactivation inhibited their motility, reduced tumor growth, and inhibited metastasis formation in xenograft models, with an associated downregulation of the miRNA oncogenic target genes, such as c-myc, E2F3, CDK6, and TGIF2. Most importantly, the involvement of miR-148a, miR-34b/c, and miR-9 hypermethylation in metastasis formation was also suggested in human primary malignancies because it was significantly associated with the appearance of lymph node metastases. These findings indicate that DNA methylation-associated silencing of tumor suppressor miRNAs contributes to the development of human cancer metastasis.

miRNAs are highly expressed in metastatic breast cancer cells and positively regulate cell migration and invasion (Ma et al. 2007). Overexpression of miR-10b in otherwise nonmetastatic breast tumors initiates robust invasion and metastasis. Expression of miR-10b is induced by the transcription factor Twist, which binds directly to the putative promoter of miR-10b (MIRN10B). The level of miR-10b expression in primary breast carcinomas correlates with clinical progression

suggesting the workings of an undescribed regulatory pathway, in which a pleiotropic transcription factor induces expression of a specific miRNA, which suppresses its direct target and in turn activates another pro-metastatic gene, leading to tumor cell invasion and metastases.

miR-101 has been shown to inhibit EZH2 (enhancer of zeste homolog 2), a histone methyltransferase that regulates the survival and metastasis of cancer cells, which is overexpressed in aggressive solid tumors (Varambally et al. 2008). Analysis of human prostate tumors has revealed that miR-101 expression decreases during cancer progression, paralleling an increase in EZH2 expression. One or both of the two genomic loci encoding miR-101 are somatically lost in 37.5 % of clinically localized prostate cancers and 66.7 % of those with metastatic disease. Gene for miR-101 is frequently compromised in prostate, breast, ovarian, and colon cancers, as well as specific forms of brain and lung cancers and leukemia. Diagnostic tests for levels of miR-101 might predict the likelihood that cancer will spread. Reintroduction of miR-101 into cells may cause tumors to revert to a less aggressive state.

Regulation of the epithelial–mesenchymal transition by members of the miR-200 family and the pleiotropic nature of the metastasis suppressor miR-31 are among the topics of current interest (Dykxhoorn 2010). Since the miR-200 family functions as putative tumor suppressors and represents biomarkers for poorly differentiated and aggressive cancers, restoration of miR-200 expression may have therapeutic implications for the treatment of metastatic and drug-resistant tumors (Mongroo and Rustgi 2010). miRNA-31 regulates invasion and metastasis in breast cancer in a way that patients with higher miR-31 expression or lower expression of the miR-31 target genes have prolonged survival, suggesting a new pathway for therapeutic intervention (Schmittgen 2010).

13.3.9 Role of miRNAs in Cancer Diagnosis

13.3.9.1 Cancer miRNA Signatures

A cancer miRNA signature, composed of a large portion of overexpressed miRNAs, has been identified from a large-scale miRNome analysis of samples including lung, breast, stomach, prostate, colon, and pancreatic tumors (Volinia et al. 2006). These miRNAs include some with well-characterized cancer association, such as miR-17-5p, miR-20a, miR-21, miR-92, miR-106a, and miR-155. A number of the predicted targets, including the tumor suppressors RB1 (retinoblastoma 1) and TGFBR2 (transforming growth factor, beta receptor II) genes, were confirmed experimentally. These results indicate that miRNAs are extensively involved in pathogenesis of solid cancers and support their function as either dominant or recessive cancer genes. Finding such a signature is important because it shows that many forms of cancer share common genetic pathways that become scrambled as cancer takes hold and spreads. The findings also pave the way for new approaches in diagnosis and

treatment. miRNAs themselves may one day be used as therapeutics. If miRNAs that are lost are replaced and those that are overly abundant are blocked, it may be possible to prevent some of the very earliest changes that occur in the development of cancer.

Bead-based flow cytometric miRNA expression profiling of miRNAs in samples from multiple human cancers has shown distinctive miRNA fingerprints (Lu et al. 2005). Generally there was downregulation of miRNAs in tumors compared with normal tissues. The miRNA profiles reflected the developmental lineage and differentiation state of the tumors and enabled successful classification of poorly differentiated tumors, whereas mRNA profiles were highly inaccurate when applied to the same samples. These findings highlight the potential of miRNA profiling in cancer diagnosis and to select the most appropriate treatment. Cancers in which miRNA expression profiling has been shown to be of diagnostic value include chronic lymphocytic leukemia, colorectal cancer, brain tumors, prostate cancer, and lung cancer. However, most human tumor samples for which long-term clinical records are available exist only as formalin-fixed paraffin-embedded (FFPE) specimens. Therefore, one study has demonstrated the feasibility of miRNA profiling studies in routinely processed FFPE human breast cancer specimens using fluorescence-labeled bead technology (Hasemeier et al. 2008).

13.3.9.2 Diagnostic Value of miRNA in Cancer

miRNAs have been shown to hold great potential as effective biomarkers for various cancers. Cancer biomarkers, including miRNAs, are important for cancer diagnosis as well as prognosis, anticancer drug development, and monitoring of response to therapy (Jain 2010). miRNAs are complementary to existing diagnostic and prognostic cancer biomarkers. Since the number of miRNAs that can describe a prognostic signature is less than the number of coding genes, they offer an advantage over gene expression signatures as biomarkers and only a few miRNAs need to be tested, by a less expensive technique to validate the previous results and adapt the signature for clinical use (Spizzo et al. 2008).

miRNAs can be used as tumor predisposition biomarkers in cancer screening programs. miRNAs might have an advantage over gene expression signatures as biomarkers. miRNAs that play roles in various steps of metastasis without obvious involvement in tumorigenesis, have also been identified. Understanding how these metastasis-associated miRNAs, termed metastamirs, are involved in metastasis will help identify possible biomarkers or targets for the most lethal attribute of cancer: metastasis (Hurst et al. 2009).

A 2006 workshop entitled “MicroRNA: Potential for Cancer Detection, Diagnosis, and Prognosis” sponsored by the Cancer Diagnosis Program of the NCI concluded that the usefulness of miRNAs for the detection, diagnosis, prognosis, and possible treatment of human cancer will depend on carefully designed translational studies (Tricoli and Jacobson 2007). In addition, it will require careful consideration of the best methods for sample collection, miRNA isolation, miRNA

quantitation, and data analysis. To facilitate this, there is a need to gain a better understanding of specific miRNA characteristics, such as how targeting of multiple mRNAs by a single miRNA affects data interpretation in biomarker studies and the effect of miRNA isoforms on diagnostic utility. In the therapeutic arena, there is a requirement to target the correct miRNA sites without affecting miRNA targets of similar sequence.

A key step toward understanding the function of the hundreds of miRNAs identified in animals is to determine their expression during development. Determining the expression levels of miRNAs is of great interest to researchers in many areas of biology, given the significant roles these molecules play in cellular regulation. Several molecular diagnostic technologies have been used for this purpose including PCR, locked nucleic acid (LNA), in situ hybridization (ISH), and microarrays. miRNAs are biomarkers and measurement of miRNA has diagnostic value for diseases as well, particularly cancer.

miRNAs are present in human plasma in a remarkably stable form that is protected from endogenous RNase activity. Measurement of tumor-derived miRNAs in serum or plasma is an important approach for the blood-based detection of human cancer. LNA is a conformational RNA analog that binds to RNA with unprecedented affinity and specificity, making it well suited for miRNA detection and analysis for cancer diagnostics (Stenvang et al. 2008).

13.3.9.3 Prognostic Value of miRNA in Cancer

Overexpression of miR-155 significantly downregulates the core mismatch repair (MMR) proteins, hMSH2, hMSH6, and hMLH1, inducing a mutator phenotype and microsatellite instability (MSI). Inactivation of MMR is the cause of the common cancer predisposition disorder Lynch syndrome (hereditary nonpolyposis colorectal cancer), as well as 10–40 % of sporadic colorectal, endometrial, ovarian, gastric, and urothelial cancers. An inverse correlation between the expression of miR-155 and the expression of MLH1 or MSH2 proteins is found in human colorectal cancer. A number of MSI tumors with unknown cause of MMR inactivation displayed miR-155 overexpression. These data provide support for miR-155 modulation of MMR as a mechanism of cancer pathogenesis by silencing genes that protect the genome from cancer-causing mutations (Valeri et al. 2010). Thus miR-155 expression might be an important stratification factor in the prognosis and treatment of cancer patients and provide an additional analytical test for exploring the etiology of MSI tumors when the standard tests do not provide a conclusive diagnosis.

13.3.10 miRNA-Based Cancer Therapeutics

Discovery of the involvement of miRNAs in the initiation and progression of human cancer is providing additional targets for anticancer treatments. Depending on

miRNA function and status in cancer, miRNAs are generally classified as tumor suppressors or oncomiRs, with different therapeutic approaches developed for each class (Pereira et al. 2013). OncomiRs (e.g., miR-21, miR-17-92 cluster, and miR-155) are known to downregulate tumor suppressor genes and have been reported to be overexpressed in multiple miRNA expression profiling studies. Therefore, therapeutic strategies aimed at inhibiting oncogenic miRNAs relieve repression of their targets. In contrast, tumor suppressor miRNAs (e.g., let-7 and miR-34 families, miR-15/16, and miR-143/145), mostly underexpressed in cancer, are responsible for downregulating oncogenes. In this case, therapeutic strategies based on miRNA aim to restore their normal cellular levels via exogenous administration of short double-stranded miRNA mimics with functions similar to endogenous miRNAs or DNA constructs coding for specific miRNAs.

Given the critical roles of miRNAs and epigenetics in cancer, characterizing the epigenetic regulation of miRNAs will provide novel opportunities for the development of cancer biomarkers and/or the identification of new therapeutic targets in the foreseeable future (Yang et al. 2008).

13.3.10.1 Antisense Oligonucleotides Targeted to miRNA

Inhibition of oncomiRs has been achieved by using small antisense single-stranded oligonucleotides complementary to miRNAs, functioning as miRNA antagonists, or mRNAs containing multiple target sites for a specific miRNA, acting as miRNA sponges, sequestering endogenous aberrantly overexpressed miRNAs (Bader et al. 2011). Antisense RNA oligonucleotides can be used for this purpose, but they need to be chemically modified to enable stability in serum and cellular uptake. If the delivery problem can be overcome, miRNA-based therapies would be feasible. A different approach is based on design of synthetic miRNAs to target overexpressed tumor proteins, such as HER-2 protein (Tsuda et al. 2005). A synthetic miRNA targeting HER-2 mRNA was successfully used to inhibit HER-2 protein expression in ovarian cancer cells. These studies indicate promise of miRNAs as future therapeutic targets. However, any approach to knock down a particular miRNA with antisense oligonucleotides (ASOs) will only result in partial knockdown (Sassen et al. 2008). This may represent a limitation for cancer therapies.

LNA-mediated silencing of miRNA function has been demonstrated in vitro and in vivo by systemically administered LNA-anti-miRs in rodents and primates. These findings support the potential of LNA in therapeutic intervention of cancer-associated miRNAs (Stenvang et al. 2008).

13.3.10.2 Role of miRNAs in Adoptive Immunotherapy of Cancer

NCI scientists have discovered that genetically engineering T lymphocytes with the gene miR-181a dramatically augments the function of poorly responsive human tumor-infiltrating lymphocytes and T-cell receptor (TCR)-engineered peripheral

blood lymphocytes, resulting in potent anticancer reactivity. This patented technology was the first reported use of an miRNA gene to treat disease. In a mouse model, miR-181a increased the function of self-/tumor-specific CD8+ T cells enabling effective tumor destruction in the absence of vaccination or exogenous cytokines that were otherwise essential requirements. Preclinical work on miR-181a has been completed and clinical studies are being planned. There are potential opportunities for collaborators that want to develop, evaluate, or commercialize the therapeutic use of miRNA-181a in the adoptive immunotherapy of cancer.

13.3.10.3 Restoration of Tumor Suppressor miRNAs to Inhibit Cancer

Many of these miRNAs modulate the major proliferation pathways through direct interaction with critical regulators such as RAS, PI3K/PTEN, or ABL, as well as members of the retinoblastoma pathway, cyclin-CDK complexes, or cell cycle inhibitors of the INK4 or Cip/Kip families (Bueno et al. 2008). A complex interplay between miRNAs and myc or E2F family members also exists to modulate cell cycle-dependent transcription during normal or cancer proliferation. Lack of a specific miRNA allows the expression of malignancy-promoting genes in certain types of T-cell cancers. An miRNA-rich chromosomal region was identified in mice, which is frequently lost in T-cell malignancies. This particular region encodes about 12 % of all genomic miRNAs. One particular miRNA, miR-203, was silenced by both genetic and epigenetic mechanisms in several mouse and human blood cell malignancies, including CML and certain ALLs. Transcriptional silencing of miR-203, which functions as a tumor suppressor, caused upregulation of the oncogene ABL1 and the BCR–ABL1 oncogenic fusion protein. Restoration of miR-203 resulted in reduction of ABL1 and BCR–ABL1 and in decreased proliferation of malignant cells. This may be particularly beneficial for patients who are resistant to small-molecule kinase inhibitors like imatinib mesylate (Gleevec), as resistant isoforms of ABL and BCR–ABL should contain the target site for miR-203 and are likely to respond to restored miR-203 function. The ability of miRNAs to modulate these proliferation pathways may have relevant implications not only in physiological or developmental processes but also in cancer therapy.

13.3.10.4 Delivery Strategies for miRNA Modulators in Cancer

Table 13.2 shows delivery strategies for miRNA inhibition therapies in cancer, and Table 13.3 shows strategies to restore miRNA levels in cancer for inhibiting cancer growth.

miRNA modulators can be delivered either locally by direct injection at the site of action or systemically by injection into the blood circulation to reach cancer cells. One of the advantages of local delivery is reduction of exposure of miRNA modulators to nuclease degradation. It increases bioavailability of miRNA modulator at target sites and minimizes contact with nontarget tissues. However, this

Table 13.2 Delivery strategies for miRNA inhibition therapies in cancer

Approach	Vector type	miRNA	Cancer	Administration
Antagomirs	No vector	miR-17-5p	Neuroblastoma	Intratumoral
		miR-221/222	Prostate	Intratumoral
		miR-10b	Breast	Systemic
Locked nucleic acids	No vector	miR-19a	Breast	Systemic
Viral vector	Lentivirus	miR-494	Breast	Systemic
Nonviral vector	Atelocollagen	miR-34a	Colorectal	Subcutaneous/ peritumoral
Targeted delivery	Peptide-targeted nanoparticles	miR-132	Breast	Systemic

Modified from Pereira et al. (2013)

Table 13.3 Delivery strategies for miRNA replacement to inhibit cancer growth

Approach	Vector type	miRNA	Cancer	Administration
Viral vector	Lentivirus	let-7g	Lung	Intratracheal
		Let-7c	Prostate	Intratumoral
		miR-15a-16-1	Prostate	Intratumoral
	Adenoviruses	let-7a	Lung	Intranasal
	Adeno-associated viruses	miR-26a	Liver	Systemic
Nonviral vector	Cationic liposomes	miR-7	Lung	Intratumoral
		miR-143	Colorectal	Intratumoral/systemic
		miR-34a; miR-143/145	Pancreas	Systemic
	Neutral liposomes	miR-34a; let-7b	Lung	Intratumoral/systemic
		miR-34a	Lymphoma	Systemic
	Polyethyleneimines	miR-145; miR-33a	Colorectal	Intratumoral/systemic
		miR-145	Lung	Intratumoral
		miR-145	Glioblastoma	Intratumoral
	Atelocollagen	miR-34a	Colorectal	Subcutaneous/ peritumoral
		miR-16	Prostate	Systemic
Targeted delivery	Single-chain variable fragment-targeted nanoparticles	miR-34a	Melanoma	Systemic

Modified from: Pereira et al. (2013)

approach is limited to a few cancers such as eye and brain tumors and sarcomas. Systemic delivery has a much broader application as it provides a simple route for miRNA modulator administration to all tumors supplied by blood vessels. However, challenges to this approach are in vivo barriers in addition to nuclease degradation.

In 2010, Marina Biotech Inc. reported results from in vivo studies in rodent cancer models focused on effective delivery of an miRNA mimetic using its Di-Alkylated Amino Acid (DiLA2) delivery system. Organ and tumor distribution studies demonstrated up to a 100-fold increase in miRNA copies per tumor cell as compared to baseline levels. Similar increases in the miRNA levels were noted in the liver, lung,

and heart after systemic administration of the mimetic formulated in DiLA2-based liposomes. Moreover, delivery of the miRNA mimetic in DiLA2 liposomes demonstrated ~60 % knockdown of mRNA for two genes whose downregulation is the intended target of the miRNA mimetic. Marina Biotech plans to expand its therapeutic reach beyond its proprietary UsiRNA-based therapies to include both miRNA mimetics and antagomirs. Its broad delivery capability which includes both the DiLA2 and SMARTICLES® technologies combined with CRN technology for stabilizing single-stranded oligonucleotides creates a formidable drug discovery engine for the development of miRNA-based therapeutics.

13.4 Current Status and Future Prospects of miRNA in Oncology

miRNA is the fastest expanding area of research in RNAi. Of the numerous miRNA inhibitors in development for therapeutic applications, currently only a few are for cancer. miRNA inhibitor miR-21, a nucleotide analog with phosphorothioate backbone, is in preclinical development for hepatocellular carcinoma. miRNA mimics—miR-34, miR-16, and let-7—are chemical modifications with lipid-based vehicles, which are at the lead optimization for cancer. The activity in this area, however, is expanding rapidly.

Antagonization of miRNAs would create a new frontier for pharmaceutical research where an entire disease pathway, rather than a single disease, is targeted for intervention. The unique mechanism of action of miRNA has a potential impact on development of therapeutics for a broad range of diseases including cancer. ASO approaches for inhibiting miRNA function and siRNA-like technologies for replacement of miRNAs are currently being explored as tools for uncovering miRNA biology and as potential therapeutic agents. There are >100 registered clinical trials evaluating the use of miRNAs as disease biomarkers in cancer. There has been remarkable increase in the number of US and European miRNA-related patent application filings. The next few years should see significant progress in our understanding of miRNA biology and the advancement of the technology for therapeutic modulation of miRNA activity. Many new roles for individual miRNAs in various diseases are likely to emerge in the near future.

ASOs readily inhibit miRNAs far more reliably than they do mRNAs, and the unique properties of Argonaute proteins allow the use of remarkably short (15 nucleotides long) ASOs, which are now in clinical trials, and 8-nucleotide-long versions show promise in nonhuman primates. However, effective and safe delivery of anti-miRNA drugs remains difficult for many cell types such as brain and muscle. Therefore, treatment of diseases with anti-miRNA oligonucleotides will require the development of novel modification, conjugation, or formulation strategies. It is hoped that anti-miRNA therapeutic strategies will soon focus on a small number of "platform" technologies that will enable rapid and safe development from discovery to effective drug (Broderick and Zamore 2011).

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Chapter 14
Biotechnology in Drug Discovery and Development for Cancer

14.1 Introduction

Traditional drug discovery methods were slow and labor-intensive and limited the number and chemical diversity of the compounds and targets that can be screened in a given assay. Even though many thousands of distinct chemical structures exist, it is not unusual for screening utilizing this approach to be terminated at the end of several years with no lead compounds identified, having examined only a small fraction of available compounds. This limitation of speed and scale often restricts both the quality and quantity of lead compounds available for further testing and development, thereby hindering drug discovery. In an improvement of this approach, several "hits" are produced as a result of high-throughput screening (HTS). Hit-to-lead stage has been added to the drug discovery. Multiple parameters are optimized in parallel to produce leads with a balanced profile of biological and physicochemical properties. New technologies are playing an increasing role in this process. It is desirable to have multiple series in hit optimization so that more than one series is available for lead optimization (Jain 2009). Important biotechnologies that have been used for improving drug discovery are listed in Table 14.1. Most of the new drug development involves biotherapeutics.

14.2 Anticancer Biotherapeutics

Classification of anticancer biotherapeutics is shown in Table 14.2. Separate chapters deal with most of these, whereas some technologies relevant to drug discovery are described in this chapter.

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_14,
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Table 14.1 Biotechnologies for improving drug discovery

Biotechnologies
Biomarkers
Bioinformatics
Molecular diagnostic technologies
Microarrays and biochips
Monoclonal antibodies (MAbs)
Nanobiotechnology
Omics technologies
Genomics: sequencing
Proteomics
Metabolomics and metabonomics
RNA interference (RNAi)
Synthetic biology
Systems biology

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Table 14.2 Classification of anticancer biotherapeutics

Classification
Anticancer agents with multiple components
Antibody–drug conjugates
Antisense therapy
Antisense oligonucleotides
Cancer immunotherapy
Cancer vaccines
Cell therapy
Cell-based cancer vaccines
Stem cell-based anticancer therapies
Drugs targeting ion channels
Drugs targeting multiple targets and pathways involved in cancer
Gene therapy
Monoclonal antibodies (MAbs)
Microbial anticancer agents
Bacterial oncolysis
Viral oncolysis
MicroRNA (miRNA)-based therapy
Nanobiotechnology-based cancer therapy
Nanobiotechnology-based drug discovery
Nanoparticle (NP) formulations of anticancer agents
NP–MAb conjugates
NPs combined with other anticancer agents
Recombinant proteins
RNA interference (RNAi)
Short interfering RNA (siRNA)-based therapy

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14.3 Role of Biomarkers in Anticancer Drug Discovery and Development

14.3.1 Biomarkers for Drug Discovery in Oncology

Among the current applications of biomarkers, those for drug discovery and development are one of the most important. Biomarkers are used in new approaches to remedy the shortage of new drugs and the current lengthy and expensive process of drug development. Biomarkers can be used to predict and confirm target binding; to determine the mechanism of action of a drug, pharmacokinetics, and toxicity; and to monitor disease status, stratify patients, and determine treatment efficacy in clinical trials. Increasing identification of signaling pathways and molecular targets involved in human cancers is improving the understanding of molecular mechanisms for carcinogenesis and development of drug resistance, which will provide new insights into drug discovery as well as design targeted therapies for cancer. Use of "omics" technologies, particularly proteomics, is providing new targets for anticancer drugs.

14.3.2 Molecular Targets of Anticancer Drugs as Biomarkers

Decreased expression of the molecular target for an antisense drug, GTI-2040 (Lorus Therapeutics Inc.), was used as a biomarker in a phase II breast cancer clinical trial (Juhasz et al. 2006). A rapid and practical method was developed to measure ribonucleotide reductase R2 (also known as M2), a malignant determinant that is the molecular target of GTI-2040. Rapid and dramatic reduction in expression of the gene for the R2 component of ribonucleotide reductase was demonstrated in tumor-biopsy tissue following treatment with GTI-2040 in combination with capecitabine. An approximately 25-fold decrease in R2 was seen as early as one day after the start of GTI-2040 treatment. This finding, in conjunction with an observed clinical response of 6 months duration, was paralleled by an observed reduction of the R2 target in circulating WBCs. This decrease suggests a potential utility of WBCs as a "surrogate" tissue for measuring this cancer biomarker, which may also be useful for evaluating the activity of GTI-2040 in downregulating target gene expression in patients for whom tumor biopsy is not possible. M65 ELISA, which quantitates different forms of circulating cytokeratin-18, and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) as pharmacodynamic biomarker assays have favorable performance characteristics for further investigation in clinical trials of anticancer agents that induce tumor apoptosis/necrosis or knockdown of the antiapoptotic protein XIAP (Cummings et al. 2006).

14.3.3 Biomarkers for Drug Development in Oncology

Response evaluation criteria in solid tumors (RECIST) guidelines have been in use for evaluation of results of treatment of cancer in clinical trials since 2000. RECIST is a combined assessment of all existing lesions, characterized by target lesions (to be measured) and nontarget lesions, which is used to extrapolate an overall response to treatment. There are some limitations to this approach. For example, trial eligibility and endpoints based solely on tumor regression are not applicable to the majority of the clinical manifestations of prostate cancers representing all clinical states. PSA-based eligibility and outcomes under RECIST conflict with established reporting standards for the states of a rising PSA and metastatic disease in a castrated patient. The clinical manifestations of prostate cancer across multiple disease states are not addressed adequately using the eligibility criteria and outcome measures defined by RECIST. Treatment effects can be described more precisely if eligibility criteria are adapted to the clinical question being addressed and clinical state under study, focusing on the duration of benefit defined biochemically, radiographically, and/or clinically.

Several candidate biomarkers have the potential to serve as a guide to the development of safer and more efficacious drugs. Biomarkers will provide a better dimension of assessment of response to treatment. Various clinical areas are expected to move toward adoption of biomarkers at different rates, but oncology is expected to have the largest gains from biomarkers over the next 5–10 years. The reasons for this are as follows:

- Cancer is a genetic disease.
- Cancer specimens are readily available for testing.
- Cancer is life-threatening, creating an urgency to find a treatment solution and a greater willingness to accept risk.
- Patients are very sick and often receive a number of drugs in a short time.
- Patients have low clinical response rates to treatment.

Biomarkers hold promise for optimization in dosing, adverse event prediction, efficacy evaluation, lead prioritization, and mechanism-of-action profiling of drug candidates. In addition, oncology has a strong pipeline of drugs in development and a number of smaller companies engaged in innovative research. Already, physicians have access to tests that help identify breast cancer patients at low risk for recurrence, who may not benefit from systemic adjuvant therapy. Identifying suitable biomarkers of biologic activity is important when assessing novel anticancer compounds such as angiogenesis inhibitors to optimize the dose and schedule of therapy.

14.3.3.1 Molecular Imaging of Tumor as a Guide to Drug Development

The field of molecular imaging offers potential to deliver a variety of probes that can image noninvasively drug targets, drug distribution, cancer gene expression, cell surface receptor or oncoprotein levels, and biomarker predictors of prognosis,

therapeutic response, or failure. Some applications are best suited to accelerate preclinical anticancer drug development, whereas other technologies may be directly transferable to the clinic. Noninvasive in vivo imaging is being applied to specific preclinical or clinical problems to accelerate progress in the field. By enabling better patient selection and strategies for monitoring treatment, molecular imaging will likely reduce the future cost of drug development in oncology.

For decades anatomic imaging with CT or MRI has facilitated drug development in medical oncology by providing quantifiable and objective evidence of response to cancer therapy. In recent years metabolic imaging with ^{18F}fluorodeoxyglucose-positron emission tomography (PET) has added an important component to the oncologist's armamentarium for earlier detection of response that is now widely used and appreciated. These modalities along with ultrasound and optical imaging (bioluminescence, fluorescence, near-infrared imaging, multispectral imaging) are being used increasingly in preclinical studies in animal models to document the effects of genetic alterations on cancer progression or metastases, the detection of minimal residual disease, and response to various therapeutics including radiation, chemotherapy, or biologic agents.

14.3.3.2 Use of PET to Assess Response to Anticancer Drugs

Thymidylate synthase is a key enzyme for the de novo synthesis of DNA and as such a target for anticancer drug development. ^{18F}FLT-PET can be used to measure thymidylate synthase inhibition as early as 1–2 h after treatment with 5-FU by a mechanism involving redistribution of nucleoside transporters to the plasma membrane (Perumal et al. 2006). PET scans can be used in clinical trials to assess whether a patient's tumor is responding to treatment even if it is not shrinking in size as shown on CT scan. Only PET could determine if tumor cells were undergoing apoptosis and the drug was having the desired effect. Changes in tumor size may take a long time. PET technology can detect HER2 positivity as well as biomarkers of hypoxia and angiogenesis (integrin ανβ3 and vascular endothelial growth factor [VEGF]). Specific molecular pathways may also be analyzed, e.g., for abnormal PI3-kinase/AKT activity, by measuring the accumulation of 11C acetate by PET and that of the RAS/RAF/MEK/ERK pathway by changes in labeled choline.

Of the molecular biochemical alterations that occur during apoptosis, activation of caspases, notably caspase-3, is the most attractive for developing specific in vivo molecular imaging probes. An isatin-5 sulfonamide, ^{18F}ICMT-11, has subnanomolar affinity for activated capsase-3 and high metabolic stability and binds to a range of drug-induced apoptotic cancer cells in vitro and in vivo by up to twofold at 24-h posttreatment compared to vehicle treatment (Nguyen et al. 2009). The increased signal intensity in tumors after drug treatment, detected by whole body in vivo microPET imaging, is associated with increased apoptosis. Thus ^{18F}ICMT-11, as a caspase-3-/caspase-7-specific PET imaging radiotracer for the assessment of tumor apoptosis, is useful in anticancer drug development and the monitoring of early responses to therapy.

14.3.3.3 VEGF-PET Imaging for Analysis of Angiogenic Changes Within a Tumor

Noninvasive imaging of angiogenesis could ease the optimization of antiangiogenesis treatments for cancer. A study has evaluated the role of VEGF-PET as a biomarker of dynamic angiogenic changes in tumors following treatment with the kinase inhibitor sunitinib (Nagengast et al. 2010). The effects of sunitinib treatment and withdrawal on the tumor were investigated using the new VEGF-PET tracer ^{89}Zr-ranibizumab as well as ^{18}F-FDG PET and ^{15}O-water PET in mouse xenograft models of human cancer. The imaging results were compared with tumor growth, VEGF plasma levels, and immunohistologic analyses. In contrast to ^{18}F-FDG and ^{15}O-water PET, VEGF-PET demonstrated dynamic changes during sunitinib treatment within the tumor with a strong decline in signal in the tumor center and only minimal reduction in tumor rim, with a pronounced rebound after sunitinib discontinuation. VEGF-PET results corresponded with tumor growth and immunohistochemical vascular and tumor biomarkers. These findings highlight the strengths of VEGF-PET imaging to enable serial analysis of angiogenic changes in different areas within a tumor.

14.3.3.4 Use of MRI to Assess Response to Anticancer Drugs

Dynamic contrast-enhanced (DCE)-MRI uses T2-captured images immediately after contrast injection (evaluating perfusion) and T1 images over a few minutes to examine extravasation of contrast for evaluating blood volume within tumor and microvascular permeability (Hylton 2006). Measurable changes in tumor perfusion or vascular permeability provide pharmacodynamic evidence of antiangiogenic effect and may provide information early in a treatment course about likely response to such therapies. DCE-MRI seems to be a useful indicator of drug pharmacology, but additional research is needed to determine if it is a suitable biomarker for predicting clinical activity.

14.3.3.5 Biomarkers in Plucked Hair for Assessing Cancer Therapy

There is a need for a technology that can quantitatively assay multiple proteins from a single hair follicle while preserving the morphology of the follicle. For proteomic profiling, the technology should be less labor-intensive, with a higher throughput, more quantitative, and more reproducible than immunohistochemistry. Layered expression scanning of hair (LES-hair) has been used to detect the levels and localization of proteins in plucked hair follicles (Traicoff et al. 2005). These proteins included cleaved caspase-3, Ki-67, and the phosphorylated forms of c-Kit, epidermal growth factor (EGF) receptor, and VEGF receptor. LES-hair provides a research tool for studying the basic biology of plucked hair follicles and has potential clinical applications such as using plucked hair follicles as a surrogate tissue to monitor pharmacodynamic effects of targeted cancer therapies.

The "plucked hair" biomarker program (Epistem Inc.) has evolved from the discovery of the link between the stem cells in the small intestine and the hair follicle. This biomarker has been developed as a noninvasive tool to measure drug effects on adult epithelial stem cells and tissues. Plucked human hairs are analyzed for the corresponding changes in gene expression at various times during cancer treatment. Gene expression changes in hairs can provide pharmaceutical companies with a measure of drug exposure, toxicity, dose/schedule, and patient selection in preclinical and clinical drug development. By comparing the gene sets linked to tumors as well as drug exposure and toxicity, it may be possible to eventually use the hair biomarker approach for evaluating the effectiveness of new cancer treatments. This approach also has the potential to offer oncologists a simple means to more effectively treat cancer patients.

14.3.3.6 Role of Biomarkers in Phase I Clinical Trials of Anticancer Drugs

A new model has been suggested of early clinical trial design involving patient selection through predictive biomarkers for selected molecularly targeted agents (Carden et al. 2010). This model can maximize the chances of patient benefit and the yield of biological and clinical information as well as direct subsequent clinical trials. Ultimately, this may result in a new paradigm of drug development, focused on patients with tumors with the same oncogenic molecular abnormalities rather than focused on patients with tumors from the same anatomical site or similar histopathology. Such biomarkers, predicting response to molecular-targeted agents, have the potential for selecting patients for these trials who are more likely to benefit from the treatment. This may facilitate early experience of and steps toward clinical qualification of predictive biomarkers, generate valuable information on cancer biology, and enable development of personalized anticancer therapy. New models of phase I study design of cancer that incorporate patient selection based on predictive biomarkers have the potential to accelerate anticancer drug development for many molecular-targeted novel agents. Indeed, it is probable that the early identification of such predictive biomarkers will improve the odds of eventual drug registration.

A genomics-based approach has been used to identify pharmacodynamic biomarkers for a cyclin-dependent kinase inhibitory drug, R547 (Roche), which is a potent cyclin-dependent kinase inhibitor with a potent antiproliferative effect at pharmacologically relevant doses and is currently in phase I clinical trials (Berkofsky-Fessler et al. 2009). Using preclinical data derived from microarray experiments, they identified pharmacodynamic biomarkers for further testing in blood samples from patients in clinical trials. The selection of candidate biomarkers was based on several criteria: relevance to the mechanism of action of R547, dose responsiveness in preclinical models, and measurable expression in blood samples. They identified 26 potential biomarkers of R547 action and tested their clinical validity in patient blood samples by quantitative real-time polymerase chain

reaction (PCR) analysis. Based on the results, eight genes (FLJ44342, CD86, EGR1, MKI67, CCNB1, JUN, HEXIM1, and PFAAP5) were selected as dose-responsive pharmacodynamic biomarkers for phase II clinical trials.

14.4 Role of Biochips/Microarrays in Drug Discovery

Biochip is a broad term indicating the use of microchip technology in molecular biology and can be defined as arrays of selected biomolecules immobilized on a surface. For practical purposes, the terms "DNA microarray" and "DNA chip" are used as synonyms although there are some technical differences. Biochip indicates the use of microchip technology in molecular biology and can be defined as arrays of selected biomolecules immobilized on a surface. An array is an orderly arrangement of samples. The sample spot sizes in microarray are usually less than 200 μm in diameter. DNA microarray comprises DNA probes formatted on a microscale plus the instruments needed to handle samples (automated robotics), read the reporter molecules (scanners), and analyze the data (bioinformatic tools). Biochip technologies and applications are described in detail in a special report on this topic (Jain 2013).

Microarrays/biochips have a significant impact on the drug discovery process. DNA microarrays enable treatment of cells with compounds and comparison of resulting patterns of gene expression with patterns previously obtained when treating cells in known ways, thereby identifying which proteins or targets the compound is altering. Such in vitro target identification should greatly improve the inefficient methods by which the drugs are currently developed. Because animal testing of compounds is expensive, time-consuming, and has other negative aspects, DNA microarrays are likely to improve the efficiency of drug discovery by supplementing the information obtained by traditional animal testing. High-throughput gene expression analysis is playing an important part in the drug discovery process in a genomic-oriented atmosphere. This requires an ability to rapidly survey and compare gene expression levels between reference and test samples. In this setting, microarray technology is exploiting collections of known sequences to pinpoint drug targets.

14.4.1 SmartChip for Cancer Drug Discovery

The University of Southern California (USC) Center for Molecular Pathways and Drug Discovery is using WaferGen Biosystems' SmartChip system to discover and validate biomarkers for pancreatic and colorectal cancer to expedite therapeutic development. USC is attempting to validate molecular signatures based on a large number of patient samples rapidly and cost-effectively to enable more targeted clinical trials. The SmartChip system comprises 5,184-well consumable chips preloaded with target-specific primers; a single-sample or multi-sample nanodispenser

for dispensing samples and master mix onto a SmartChip panel under vacuum conditions; and the SmartChip cycler, which performs PCR thermal cycling and data collection.

14.4.2 Use of Microarrays in Clinical Trials

Various applications of microarrays/biochips described in preceding chapters can be useful in clinical trials. Gene expression microarrays are used in oncology clinical trials. Several multigene markers that predict relapse in cancer more accurately than classical clinicopathologic features have been developed. An example is the 21-gene assay that was developed specifically for patients with estrogen receptor (ER)-positive breast cancer and has been shown to predict distant recurrence more accurately than classical clinicopathologic features in patients with ER-positive breast cancer and negative axillary nodes treated with adjuvant tamoxifen; validation studies in this population led to its approval as a diagnostic test. In a similar population, it also may be used to assess the benefit of adding chemotherapy to hormonal therapy. Other validation studies indicate that it also predicts the risk of distant and local recurrence in other populations with ER-positive disease, including node-negative patients receiving no adjuvant therapy and patients with positive axillary nodes treated with doxorubicin-containing chemotherapy. The Trial Assigning Individualized Options for Treatment (TAILORx) is a multicenter trial that integrates the 21-gene assay into the clinical decision-making process and is designed to refine the utility of the assay in clinical practice and to provide a resource for evaluating additional molecular biomarkers as they are developed in the future (Sparano and Paik 2008).

14.4.3 Reverse-Phase Protein Microarrays

Reverse-phase protein microarrays (RPMA) are a technology platform designed for quantitative, multiplexed analysis of specific phosphorylated, cleaved, or total (phosphorylated and non-phosphorylated) forms of cellular proteins from a limited amount of sample. This class of microarray can be used to interrogate tissue samples, cells, serum, or body fluids. RPMA were previously a research tool; now this technology has graduated to use in research clinical trials with clinical grade sensitivity and precision. RPMA have been used for multiplexed signal pathway analysis in therapeutic monitoring, biomarker discovery, and evaluation of pharmaceutical targets (Mueller et al. 2010).

Reverse-phase protein arrays have been used to examine phosphorylation status of proteins in a panel of breast cancer cell lines and showed distinct pathway activation differences between different subtypes that were not obvious from previous gene expression studies (Boyd et al. 2008). The authors showed that basal levels of

phosphorylation of key signaling nodes may be useful for predicting response to selective inhibitors of phosphatidylinositol 3-kinase and mitogen-activated protein (MAP) kinase/extracellular signal-regulated kinase. Finally, they showed that reverse-phase protein arrays enable the parallel analysis of multiple pharmacodynamic biomarkers of response to targeted kinase inhibitors and that inhibitors of epidermal growth factor receptor (EGFR) and MAP kinase/extracellular signal-regulated kinase kinase result in compensatory upregulation of the phosphatidylinositol 3-kinase/Akt signaling pathway.

14.4.4 Laboratories-on-a-Chip

Microfluidic systems and nanoporous materials enable construction of miniature "laboratories-on-a-chip" that might contain up to a million test chambers, each capable of screening an individual drug. Such biochips can be used to screen candidate compounds to find drugs to overcome anticancer drug resistance by deactivating the pumps in cell membranes that remove chemotherapy drugs from tumor cells, making the treatment less effective. The biochips could dramatically increase the number of experiments that are possible with a small amount of protein.

14.5 Role of Cells in Drug Discovery

Development of cells as therapeutics was described in Chap. 10. Cells are involved in the therapeutic effects of many drugs and can be manipulated by drugs. An important application is use of cell lines for drug screening. More than 60 cell lines have been used to screen >16,000 anticancer compounds of which ~300 were approved by the FDA.

14.5.1 Role of Stem Cells in Therapeutic Effects of Drugs

Some drugs exert their beneficial effects through progenitor and stem cells. One example is drug-induced neurogenesis. Chronic treatment with antidepressants as well as electroconvulsive therapy increases neurogenesis in the adult hippocampus. This increase in the production of new neurons may be required for the behavioral effects of antidepressants. A reporter mouse line has been generated, which allows identification and classification of early neuronal progenitors and further enables accurate quantitation of changes induced by neurogenic agents in these distinct subclasses of neuronal precursors (Encinas et al. 2006). This line was used to demonstrate that the selective serotonin reuptake inhibitor antidepressant fluoxetine does not affect division of stem-like cells in the dentate gyrus but increases symmetric

divisions of an early progenitor cell class. The findings suggest that the fluoxetine-induced increase in new neurons arises as a result of the expansion of this cell class. This finding defines a cellular target for antidepressant drug therapies.

14.5.2 Stem Cells for Drug Discovery

Stem cells have application in all stages of the drug discovery pathway from target identification through to toxicology studies (Hook 2012).

14.5.2.1 Target Identification

Through directed differentiation of stem cells to particular lineages in vitro, it is possible to study gene expression patterns and dissect the molecular mechanisms of lineage commitment, thereby identifying possible candidate proteins for therapeutic intervention. Coupling this with genetic modification has greatly aided gene function studies. In particular, gene targeting through homologous recombination has revolutionized the study of many biologic systems. Mouse embryonic stem cells (ESCs) and mice with a variety of modifications, such as null and point mutations, chromosomal rearrangements, large deletions, and inserted reporter genes, have enabled detailed analysis of gene function in vitro and in vivo. Although possible, gene targeting has proved more difficult in human embryonic stem cells (hESCs). However, advances in technology, such as the use of zinc finger nucleases, will enable generation of knockout hESCs. The ability to generate disease-specific induced pluripotent stem cells (iPSCs) extends target identification studies to the study of disease progression and pathology in vitro.

14.5.2.2 High-Throughput Screening

For application in drug discovery, HTS is an important way of identifying small molecules that could influence stem cells to proliferate or differentiate. HTS involves the testing of large numbers (tens of thousands to a million or more) of small molecular weight molecules to rapidly identify those that inhibit or activate a particular pathway of interest—in this case, stem cell growth and development (Rubin 2008). Current methods of drug screening rely largely on the use of recombinant transformed cell lines that express the target of interest (e.g., a G protein-coupled receptor) but otherwise are not directly relevant to the disease being studied. The use of primary cells is desirable since they are physiologically relevant. However, they are relatively difficult to obtain and since they can only be passaged a few times before senescence or death it is technically challenging to obtain cells in sufficient numbers for HTS applications. Additionally, variability between donors and in preparation of cell batches can lead to inconsistent results. Stem cells offer

an attractive alternative to primary cells and recombinant cell lines as they can be propagated for prolonged periods of time, can be cryopreserved, and can differentiate to physiologically relevant cell types. Therefore, large batches of undifferentiated or differentiated cells can be generated for use in a series of experiments or screens. Furthermore, iPSCs now offer the opportunity to generate disease-specific somatic cells and to rapidly generate panels of stem cells with a range of genetic phenotypes, enabling genetic effects on drug performance to be studied.

14.5.2.3 ESCs as Source of Models for Drug Discovery

ESCs will become a source of models for a wide range of adult differentiated cells, providing that reliable protocols for directed differentiation can be established. Stem cell technology has the potential to revolutionize drug discovery, making models available for primary screens, secondary pharmacology, safety pharmacology, metabolic profiling, and toxicity evaluation. Models of differentiated cells that are derived from mouse ESCs are already in use in drug discovery and are beginning to find uses in high-throughput screens. Before analogous human models can be obtained in adequate numbers, reliable methods for the expansion of human ESC cultures will be needed. For applications in drug discovery, involving either species, protocols for directed differentiation will need to be robust and affordable. Current challenges and future opportunities in relation to the use of stem cell technology in drug discovery have been explored by use of both mouse and human models. Challenges that need to be overcome before ESCs become a regular source of cell culture models for drug discovery include the following (Pouton and Haynes 2007):

- Establishment of universal methods for the expansion of hESCs in chemically defined media in the absence of feeder layers
- Improved methods for the control for differentiation leading to cultures with reduced heterogeneity
- Establishment of panels of human ESCs with disease-related polymorphisms
- An understanding of differences between various ESC lines
- Need for developing methods to facilitate immobilization of specialized cells, e.g., neurons, so that synapses and neurotransmitter transporters can be studied
- Improved strategies for identifying specified living cells in a mixed population to enable high-content functional assays in high throughput

14.5.2.4 Advantages and Limitations of the Use of Stem Cells for Drug Discovery

Advantages of using stem cells for drug discovery are as follows:

- The wide range of cell types and tissues that develop from stem cells represent a biologic system that mimics many of the complex interactions of the cells and tissues of the body and as such provides an attractive screening tool.

- Stem cell banks can be used for developing in vitro assays that could have wide applications in the pharmaceutical, cosmeceutical, and agrochemical industries. This can reduce the need for animal testing and to increase the efficiency and reduce the costs of developing safe and effective drugs and chemicals.
- hESCs can potentially be engineered to create in vivo models of human disease for drug development as a superior alternative to current mouse models. For example, brain neurons derived from hESCs might be engineered to develop the characteristics of Alzheimer's disease and used to discover effective drug treatments.

Use of animal-derived cells or tissues to develop selective drugs has its limitations since activity in animals or animal models does not always translate into efficacy in humans. The development of human cell-based models will be important for evaluating novel targets and compounds in a close-to-physiological environment. Stem cells provide this opportunity as they differentiate to produce various cell types. Pluripotent ESCs can be stimulated to differentiate into precursors of cells of various organs by using some small molecules, and the same molecules could have potential therapeutic applications for treatment of disorders of these organs. Important areas of applications of stem cells relevant to drug discovery are as follows (Sartipy et al. 2007):

- Identification and evaluation of various trophic factors
- Identification of novel receptors, ligands, and signaling pathways
- Gene expression profiling
- Cellular assay design for screening
- Evaluation of pharmacological or toxicological effects of new medicines. A variety of different hESC types can be used in drug screening and toxicology testing.

The pharmaceutical industry's use of stem cells for screening purposes is still under evaluation with the options available from either fetal or adult somatic stem cells of restricted potency and self-renewal capacity compared with ESCs which provide pluripotency and unlimited growth potential. ESC-based systems increase the amount and quality of biological information obtained from screens. ESCs provide a more flexible, alternative source of cells for many current operative screening technologies. Functional gene validation frequently trades off clinical relevance for screening capacity, whereas ESC-based assays accommodate both. Pivotal to the utilization of stem cells in all these contexts however is a requirement to control the mechanism of self-renewal and being able to harness the inherent pluripotent phenotype.

Widespread use of stem cells for drug discovery process will depend on the development of robust, reproducible methods to culture stem cells and to direct their differentiation into specific lineages. Discovery and optimization of stem cell differentiation are technically challenging because of several variables, e.g., choice of growth factors or cell substrates and the optimal 3D configuration of cells.

14.5.3 Targeting Cancer Stem Cells to Screen Anticancer Drugs

Screens for agents that specifically kill epithelial cancer stem cells (CSCs) have not been previously possible due to the rarity of these cells within tumor cell populations and their relative instability in culture. Now an approach has been described to screen for agents with epithelial CSC-specific toxicity (Gupta et al. 2009). A genetic manipulation screen keeps breast CSCs trapped in the stem cell state. Implementation of this method in a chemical screen led to discovery of compounds showing selective toxicity for breast CSCs. One compound, salinomycin, reduces the proportion of CSCs by >100-fold relative to paclitaxel, a commonly used breast cancer chemotherapeutic drug. Treatment of mice with salinomycin inhibits mammary tumor growth in vivo and induces increased epithelial differentiation of tumor cells. In addition, global gene expression analyses show that salinomycin treatment results in the loss of expression of breast CSC genes previously identified by analyses of breast tissues isolated directly from patients. This study demonstrates the ability to identify agents with specific toxicity for epithelial CSCs. It is hoped that pharmaceutical companies will use their screening method to begin to develop drugs against CSCs.

14.6 Role of Proteomics in Drug Discovery for Cancer

Proteins are targets for most drugs, and drug discovery for cancer shares the basic problems with drug discovery for other diseases. Application of proteomics to drug discovery in cancer has been discussed in detail elsewhere (Jain 2005).

14.6.1 Kinase-Targeted Drug Discovery in Oncology

The role of proteomic technologies in studying cell signaling pathways has been described in Chap. 4. The success of the Bcr–Abl kinase inhibitor imatinib (Novartis' Gleevec) in treating chronic myeloid leukemia (CML) has stimulated interest in the development of kinase-targeted therapies for cancer. Immunoaffinity/MS profiling of tyrosine phosphorylation events in cancer cells has revealed thousands of distinct phosphotyrosine sites across some several tumors and cancer cell lines. This technology has also been used to screen cell lines derived from patients with acute myeloid leukemia (AML) and has revealed increased phosphorylation of Jak3 kinase substrates. An alternative approach for identifying protein kinases that might be therapeutic targets is to determine the effects of suppressing their expression using RNAi.

Characterization of intracellular signaling pathways should lead to a better understanding of ovarian epithelial carcinogenesis and provide an opportunity to interfere with signal transduction targets involved in ovarian tumor cell growth, survival, and progression. Challenges toward such an effort are significant because many of these signals are part of cascades within an intricate and likely redundant intracellular signaling network. For instance, a given signal may activate a dual intracellular pathway (i.e., MEK1–MAPK and PI3K/Akt) required for fibronectin-dependent activation of matrix metalloproteinase. A single pathway also may transduce more than one biologic or oncogenic signal (i.e., PI3K signaling in epithelial and endothelial cell growth and sprouting of neovessels). Despite these challenges, evidence for therapeutic targeting of signal transduction pathways is accumulating in human cancer.

Many different intracellular protein kinases are components of mitogenic signaling pathways. One of the targets is oncogenic kinase B-raf, a mutant component of the Ras–Raf–Mek–MAP kinase cascade. B-raf mutations can be correlated with the sensitivity of tumor cells to Mek inhibitors. Exelixis has used high-throughput approaches to the discovery of phosphatidylinositol 3-kinase (PI3-kinase) pathway inhibitors. The Exelixis compound XL418, an inhibitor of Akt and P70S6 kinase, blocks the growth of tumor xenografts in mice. Combinations of XL418 with EGF receptor inhibitors display synergy in tumor models, with dramatic increases in levels of apoptosis. Targeting multiple points within a given signal transduction pathway shows clear evidence of increased efficacy in model systems, which suggests various rational combinations of drugs for clinical intervention in the PI3-kinase pathway.

14.6.2 Anticancer Drug Targeting: Functional Proteomic Screen of Proteases

Some of the anticancer strategies are directed against proteases that facilitate several steps in cancer progression. The major proteases are matrix metalloproteases, cathepsins, and the mast cell serine proteases. Assay of actual enzyme activity, and not mRNA levels or immunoassay of protein, is ideal for identifying proteases most suitable for drug targeting. Proteases are particularly suitable for functional proteomic screening. This has been achieved by an automated microtiter plate assay format that can be modified to enable detection of major classes of proteases in tissue samples. Results of such analyses show that matrix metalloproteases are localized to the tumor cells themselves, whereas cathepsin B is predominantly expressed by macrophages at the leading edge of invading tumors. Such an analysis also serves to identify proteases whose activity is not completely balanced by endogenous inhibitors and which may be essential for tumor progression. These proteases are logical targets for initial efforts to produce low molecular weight protease inhibitors as potential chemotherapy.

14.6.3 Small-Molecule Inhibitors of Cancer-Related Proteins

ARIAD Pharmaceuticals has pioneered the discovery and development of small-molecule drugs to inhibit cancer-related proteins and pathways. Structure-based drug design is used to create molecules that overcome resistance to existing treatments. A major focus is to identify inhibitors of protein kinases (enzymes that transfer phosphates between different proteins), which are implicated in specific cancers. AP26113 (ARIAD) is a small molecule that in preclinical studies has exhibited activity as a potent tyrosine kinase inhibitor of anaplastic lymphoma kinase and EGFR.

14.7 Role of RNAi for Drug Discovery in Cancer

14.7.1 Basis of RNAi for Drug Discovery

RNAi technology is being evaluated not only as an extremely powerful instrument for functional genomic analyses but also as a potentially useful method to develop highly specific dsRNA-based gene-silencing therapeutics. Target-based drug discovery starts with the identification of target genes and their respective protein products, which when inhibited or activated ameliorate the associated disease. RNAi is an important method for analyzing gene function and identifying new drug targets that uses double-stranded RNA to knock down or silence-specific genes. The challenge has been to reliably select an siRNA segment that can efficiently silence the gene without triggering unwanted effects. One solution is by using algorithms to select highly functional siRNA sequences and then pooling the best sequences for guaranteed gene knockdown. RNAi technology could considerably reduce the time needed for target validation and overall drug development, accelerating the drug discovery process. RNAi screening can identify high-value drug targets such as kinases involved in cell proliferation, which is relevant to cancer.

14.7.2 RNAi for Identification of Genes as Therapeutic Targets for Cancer

The ability of RNAi to provide relatively easy ablation of gene expression has opened up the possibility of using collections of siRNAs to analyze the significance of hundreds or thousands of different genes whose expression is known to be upregulated in cancer. Perhaps more important still is the possibility of using genome-wide collections of siRNAs, whether synthetic or in viral vectors, as screening tools. This has attracted much attention recently from both academic and industrial researchers. The libraries of RNAi reagents can be used in one of two ways. One is

in a high-throughput manner, in which each gene in the genome is knocked down one at a time and the cells or organism scored for a desired outcome, e.g., death of a cultured cancer cell but not a normal cell. Owing to the very large numbers of assays needed to look at the involvement of all genes in the human genome, this approach is very labor-intensive. The other approach is to use large pools of RNAi viral vectors and apply a selective pressure that only cells with the desired change in behavior can survive. The identity of the genes knocked down in the surviving cells can then be identified by sequencing the RNAi vectors that they carry. This method is being used to investigate genes involved in cancer. Both approaches show considerable promise in identifying novel genes that may make important therapeutic targets for inhibition either by conventional drug discovery methods or by RNAi itself.

Genetic screens and in vivo studies could be broadly improved by methods that allow inducible and uniform gene expression control. To achieve this, lentiviral pINDUCER series of expression vehicles were built for inducible RNAi in vivo (Meerbrey et al. 2011). Using a multicistronic design, pINDUCER vehicles enable tracking of viral transduction and shRNA or cDNA induction in a broad spectrum of mammalian cell types in vivo. This uniform temporal, dose-dependent, and reversible control of gene expression across heterogeneous cell populations is achieved via fluorescence-based quantification of reverse tet-transactivator expression. This feature enables isolation of cell populations that exhibit a potent, inducible target knockdown in vitro and in vivo that can be used in human xenotransplantation models to examine cancer drug targets.

14.7.3 Role of siRNAs in Drug Target Identification

Sets of siRNAs focused on a specific gene class (siRNA libraries) have the capacity to greatly increase the pace of pathway analysis and functional genomics. RNAi-based functional chemogenomics has been integrated into the drug discovery program at the Translational Genomics Research Institute (TGen). By applying ultrahigh-throughput transfection of agents that trigger RNAi, scientists at TGen can screen genes at the genome scale using detailed analyses of the functional responses in living cells to determine which specific gene inhibitors produce anticancer effects and therefore have therapeutic utility as drug targets. siRNA library resources at TGen were used to analyze 10,000 siRNA to identify vulnerable targets in breast cancer. Phenotype analysis of these siRNAs is helping in identifying modulators of chemotherapy.

Protein kinases are coded by more than 2,000 genes and thus constitute the largest single enzyme family in the human genome. They are important drug targets in human cancers, inflammation, and metabolic diseases. The goal is to find out which kinases are involved in proliferation and the cell cycle. The approach used by Cenix Bioscience is to prepare siRNAs against human kinases using a well-defined design algorithm. The experimental plan is to identify kinases that are expressed in HeLa

cells, define a set of siRNAs that individually knock down mRNAs corresponding to each kinase, perform the actual siRNA transfections, confirm the knockdown percentage for each gene, and then measure cellular proliferation and the mitotic index. Mitotic index is the percentage of cells undergoing mitosis and is measured by fluorescent microscopy using the number of mitotic cells divided by the total number of cells.

14.7.4 Validation of Oncology Targets Discovered Through RNAi Screens

The clinical efficacy of selective kinase inhibitors suggests that some cancer cells may become dependent on a single oncogene for survival. RNAi has been increasingly used to understand this mechanism and validate new therapeutic targets but are limited by significant off-target effects. Carefully titrated lentiviral-mediated shRNA knockdown of the EGFR has been combined with heterologous reconstitution by EGFR mutants to rigorously analyze the structural features and signaling activities that determine dependence on the mutationally activated EGFR in human lung cancer cells (Rothenberg et al. 2008). EGFR dependence is differentially rescued by distinct EGFR variants and oncogenic mutants, is critically dependent on its heterodimerization partner ErbB-3, and does not require autophosphorylation sites in the cytoplasmic domain. Quantitative "oncogene rescue" analysis enables mechanistic dissection of oncogene dependence and may provide functional validation for therapeutic targets identified through large-scale RNAi screens.

14.7.5 Challenges of Drug Discovery with RNAi

The advantages of cell-based RNAi screens over small-molecule screening for target identification include the fact that most cell types are amenable to RNAi and it is relatively easy to knock down any gene of interest. So far, every gene tested has been susceptible to RNAi. However, one of the big issues is how to make siRNAs "druggable." Some of the challenges are:

- To ensure that the candidate siRNA is appropriately stabilized in a "druggable" formulation or by chemical modifications. Stability of the RNA to exo- and endonucleases can be resolved by appropriate chemical modifications.
- Safely and successfully delivering siRNA in an acceptable and effective manner.
- Scaling up siRNA synthesis in the near term and, ultimately, manufacturing reliably and effectively.
- Cell-based RNAi assays are particularly prone to edge effects because the cells in the outer wells of the plates grow at a different rate than the cells in the inner wells. One should ignore the outer wells.

- There are problems with the "penetrance" of some RNAi screens, in which the level of green fluorescent protein (GFP) in the cells is heterogeneous, making it difficult to interpret. Actually, the expression levels of several proteins vary significantly within cells grown in culture. Therefore, the problem is not heterogeneity of the siRNA knockdown but heterogeneity of protein expression and is an artifact of the cell culture. Analysis of the data can be improved by looking at single cells rather than entire wells.

Once these issues are resolved, there is potential for rapid early-stage drug development as RNAi-based therapy development relies predominantly on documented gene sequence data and leverages a natural process.

14.8 Role of Nanobiotechnology in Drug Discovery for Cancer

Current drug discovery process needs improvement in several areas. Although many targets are being discovered through "omics" technologies, the efficiency of screening and validation processes need to be increased. Through further miniaturization, nanotechnology will improve the ability to fabricate massive arrays in small spaces using microfluidics and the time efficiency. This would enable direct reading of the signals from microfluidic circuits in a manner similar to a microelectronics circuit where one does not require massive instrumentation. This would increase the ability to do high-throughput drug screening.

The new challenges in the identification of therapeutic targets require efficient and cost-effective tools. Label-free detection systems use proteins or ligands coupled to materials, the physical properties of which are measurably modified following specific interactions. Among the label-free systems currently available, the use of metal nanoparticles offers enhanced throughput and flexibility for real-time monitoring of biomolecular recognition at a reasonable cost. Some nanobiotechnologies will accelerate target identification, whereas some nanoparticles will evolve into therapeutics.

14.8.1 Lab-on-Bead

A nanotechnology-based method for selecting peptide nucleic acid (PNA)-encoded molecules with specific functional properties from combinatorially generated libraries consists of three essential stages: (1) creation of a Lab-on-Bead library, a one-bead, one-sequence library that, in turn, displays a library of candidate molecules; (2) fluorescence microscopy-aided identification of single target-bound beads and the extraction—wet or dry—of these beads and their attached candidate molecules by a micropipette manipulator; and (3) identification of the target-binding candidate

molecules via amplification and sequencing (Gassman et al. 2010). This novel integration of techniques harnesses the sensitivity of DNA detection methods and the multiplexed and miniaturized nature of molecule screening to efficiently select and identify target-binding molecules from large nucleic acid-encoded chemical libraries and has the potential to accelerate assays currently used for the discovery of new drug candidates by screening millions of chemicals simultaneously using nanosized plastic beads. One batch of nanoscopic beads can replace the work of thousands of conventional, repetitive laboratory tests. This process could be up to 10,000 times faster than current methods. By working at nanoscale, it will be possible to screen more than a billion possible drug candidates per day as compared to the current limit of hundreds of thousands per day.

14.8.2 Nanoparticles as Anticancer Agents

14.8.2.1 Dendrimers as Drugs

Dendrimers are a novel class of three-dimensional nanoscale, core-shell structures that can be precisely synthesized for a wide range of applications. Specialized chemistry techniques allow for precise control over the physical and chemical properties of the dendrimers. They are most useful in drug delivery but can also be used for the development of new pharmaceuticals with novel activities. Polyvalent dendrimers interact simultaneously with multiple drug targets. They can be developed into novel targeted cancer therapeutics. Polymer–protein and polymer–drug conjugates can be developed as anticancer drugs. These have the following advantages:

- Tailor-made surface chemistry
- Non-immunogenic
- Inherent body distribution enabling appropriate tissue targeting
- Possibly biodegradable

Dendrimer conjugation with low molecular weight drugs has been of increasing interest recently for improving pharmacokinetics, targeting drugs to specific sites, and facilitating cellular uptake. Opportunities for increasing the performance of relatively large therapeutic proteins such as streptokinase (SK) using dendrimers have been explored in one study (Wang et al. 2007). Using the active ester method, a series of streptokinase-poly(amido amine) (PAMAM) G3.5 conjugates were synthesized with varying amounts of dendrimer-to-protein molar ratios. All of the SK conjugates displayed significantly improved stability in phosphate buffer solution, compared to free SK. The high coupling reaction efficiencies and the resulting high enzymatic activity retention achieved in this study could enable a desirable way for modifying many bioactive macromolecules with dendrimers.

Glycodendrimers are carbohydrate functionalized dendrimers for use in therapeutics, antigen presentation, and as biologically active compounds. Glycosyn, a joint venture between Starpharma Holdings and Industrial Research Ltd., will

provide manufacturing and specialized expertise in carbohydrate design, synthesis, and analysis. One of the first projects in the pipeline involves research undertaking cGMP manufacture of intermediates used in the production of Starpharma's vaginal microbicide—VivaGelT, a polyvalent dendrimer-based pharmaceutical being developed to prevent the spread of HIV/AIDS and, potentially, other sexually transmitted infections including genital herpes.

14.8.2.2 Poly-L-lysine Dendrimer as Antiangiogenetic Agent

Poly-L-lysine (PLL) sixth-generation (G6) dendrimer molecules exhibit systemic antiangiogenic activity that could lead to arrest of growth of solid tumors. Intravenous administration of the PLL-dendrimer molecules into C57BL/6 mice inhibits vascularization of tumors grown within dorsal skinfold window chambers as demonstrated by intravital microscopy (Al-Jamal et al. 2010). The in vivo toxicological profile of the PLL-dendrimer molecules shows that it is safe at the dose regime studied. The antiangiogenic activity of the PLL dendrimer is further shown to be associated with significant suppression of B16F10 solid tumor volume and delayed tumor growth. Enhanced apoptosis/necrosis within tumors of PLL-dendrimer-treated animals only and reduction in the number of CD31 positive cells are observed in comparison to protamine treatment. This study suggests that PLL-dendrimer molecules can exhibit a systemic antiangiogenic activity that may be used for therapy of solid tumors and in combination with their capacity to carry other therapeutic or diagnostic agents may potentially offer capabilities combining diagnosis with therapy.

14.8.3 Nanotechnology for Drug Design at Cellular Level

To create drugs capable of targeting some of the most devastating human diseases, one must first decode exactly how a cell or a group of cells communicates with other cells and reacts to a broad spectrum of complex biomolecules surrounding it. But even the most sophisticated tools currently used for studying cell communications suffer from significant deficiencies and typically can only detect a narrowly selected group of small molecules or, for a more sophisticated analysis, the cells must be destroyed for sample preparation. A nanoscale probe, the scanning mass spectrometry (SMS) probe, can capture both the biochemical makeup and topography of complex biological objects. SMS exploits an approach to electrospray ionization that enables continuous sampling from a highly localized picoliter volume in a liquid environment, softly ionizes molecules in the sample to render them amenable for mass spectrometric analysis, and sends the ions to the mass spectrometer (Kottke et al. 2010). The SMS probe can help map all those complex and intricate cellular communication pathways by probing cell activities in the natural cellular environment, which might lead to better disease diagnosis and drug design on the cellular level.

14.8.4 Role of Nanobiotechnology in the Future of Drug Discovery for Cancer

None of the nanoparticles available is ideal for all requirements of drug discovery for cancer. The choice may depend on the needs. QDs can be used for high-throughput cell-based studies with the advantage of multiplexing (i.e., multiple leads can be tested at the same time). However, as discussed earlier there are some limitations yet to be resolved for their use in the drug discovery studies, namely, toxicity, size variation, agglomeration, potential multiple drug attachment to a single QD, and blinking. Dendrimers are more promising for anticancer drug design as the branches allow attachment of molecules for different functions such as diagnosis and therapy for developing a multifunctional anticancer drug.

14.9 Role of Sequencing in Anticancer Drug Discovery

Next-generation sequencing, in combination with genome-wide association studies, will lead to an improved understanding of molecular mechanisms of cancer, molecular substaging of the disease, and drug response. Among various applications of sequencing, resequencing and RNA profiling are expected to have a profound effect on drug discovery and development.

14.9.1 High-Throughput Sequencing for Anticancer Drug Discovery

A unique screening strategy by HTP sequencing has been described to enable large-scale and quantitative analysis of gene matrices associated with specific disease phenotypes, which allows screening of small molecules that can specifically intervene with disease-linked gene expression events (Li et al. 2012). Application of this multitarget strategy to the problem of hormone-refractory prostate cancer, which tends to be accelerated by the current antiandrogen therapy, led to the identification of peruvoside, a cardiac glycoside, which can potently inhibit both androgen-sensitive and androgen-resistant prostate cancer cells without triggering severe cytotoxicity. It was further shown that, despite transcriptional reprogramming in prostate cancer cells at different disease stages, the compound can effectively block androgen receptor-dependent gene expression by inducing rapid androgen receptor degradation via the proteasome pathway. These findings establish a genomics-based phenotypic screening approach capable of quickly connecting pathways of phenotypic response to the molecular mechanism of drug action, thus offering a unique pathway-centric strategy for anticancer drug discovery.

14.10 Drugs Targeting Ion Channels

This subject was reviewed in a symposium on "Ion Channel Transport and Cancer (ICT)," covering the roles of ICTs in cancer cell proliferation, apoptosis, motility, and invasion and in both the generation of and the interaction of the cancer cells with the tumor environment (Pedersen and Stock 2013). Drugs from categories other than anticancer therapeutics, which are known to inhibit cancer-relevant ion channels such as the antihistamine astemizole that blocks KV10.1 channels, may inhibit metastatic disease. Most ICTs implicated in cancer are located at the plasma membrane, which is a convenient site for drug targeting. In view of the contribution of ICT dysregulation to carcinogenesis, future research should be directed at further elucidation of the mechanisms and development of therapeutic applications of this knowledge.

14.11 Drugs Targeting Multiple Cancer Pathways

14.11.1 AT13148

Deregulated phosphatidylinositol 3-kinase pathway signaling through AGC kinases including AKT, p70S6 kinase, PKA, SGK, and Rho kinase is a key driver of multiple cancers. The simultaneous inhibition of multiple AGC kinases may increase antitumor activity and minimize clinical resistance compared with a single-pathway component. The kinase inhibitor AT13148 (Astex Pharmaceuticals) is a "master-switch" experimental drug that simultaneously blocks many enzymes involved in the control of cancer cell growth and death to enable treatment of multiple cancer types including sarcoma, breast, and prostate. Most drugs block only a single enzyme, but switching off cell signals at multiple points at the same time could increase the effectiveness of this drug. Tumors will be less likely to develop resistance to this treatment. Preclinical gene expression microarray studies show that AT13148 has a mechanism of action that is distinct from other AKT inhibitors (Yap et al. 2012). Cancer Research UK is conducting phase I/II clinical trials.

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Chapter 15
Role of Biotechnology in Drug Delivery for Cancer

15.1 Introduction

Drug delivery is an important part of pharmacotherapy of cancer. Merely developing an effective anticancer agent is not enough unless it is delivered to the site of action. Traditional drug development considered formulations for different routes of administration, mostly oral or injectable. Cancer drug delivery is no longer simply wrapping the drug in new formulations for different routes of delivery. Knowledge and experience from other technologies such as nanotechnology, advanced polymer chemistry, and electronic engineering are being brought together in developing novel methods of drug delivery. The focus is on targeted cancer therapy. The newer approaches to cancer treatment not only supplement the conventional chemotherapy and radiotherapy but also aim to prevent damage to the normal tissues and overcome drug resistance. Innovative methods of cancer treatment, e.g., cell and gene therapies, require new concepts of drug delivery in cancer. New biotechnologies have contributed considerably to drug delivery in cancer and some of these have been considered in other chapters: monoclonal antibodies (MAbs) (Chap. 8), nanobiotechnology/nanooncology (Chap. 9), cell therapy (Chap. 10), gene therapy (Chap. 11), and RNAi (Chap. 12).

Details of drug delivery systems are presented in a special report on this topic (Jain 2013). A major limitation of molecular approaches to the treatment of cancer is the delivery of the drugs to the cells in solid tumors. The drug must first pass into the blood vessels of the tumor, through the vessel wall, and then into the substance of the tumor. Innovative anticancer therapies in development face similar problems. Obstacles to these steps are inherent in tumors. Drug delivery to solid tumors consists of multiple processes, including transport via blood vessels, transvascular transport, and transport through interstitial spaces. These processes are dynamic and change with time. Tumor properties are affected by multiple physicochemical factors of a drug, multiple tumor biological factors, and effects of treatments.

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_15,
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Table 15.1 Routes of drug delivery in cancer

Systemic delivery
Intravascular: intravenous and intra-arterial
Intramuscular
Subcutaneous injection or implant
Oral
Transdermal drug delivery
Nasal
Locoregional
Local delivery to the tumor or organ bearing the tumor
Intra-arterial: injection into arteries supplying the organ/region bearing the tumor
Intrathecal delivery for CNS neoplasms
Intraperitoneal delivery for tumors in the abdominal cavity
Instillation into the urinary bladder
Administration to regional lymph nodes
Pulmonary inhalation for lung tumors

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Several drug delivery systems have been investigated for possible administration of therapeutics for cancer. Protein and peptide therapeutics for cancer require special methods of delivery, including oral, nasal, and pulmonary routes. Intravenous drug delivery, antineoplastic drug implants, gene therapy, and use of MAbs are promising methods of cancer drug delivery. One unique feature of cancer is that localized delivery of drugs directly into the tumor mass is a possibility. Routes of drug delivery for cancer as shown in Table 15.1 should be considered as a background for applications of biotechnology.

15.2 Innovative Methods of Drug Delivery in Cancer

Innovative formulations for drug delivery in cancer are listed in Table 15.2.

The most important biotechnology-based category of drug delivery has already been discussed in Chap. 9 (Nanooncology). MAb-based techniques for drug delivery were described in Chap. 8. Some of the other methods will be briefly described here.

15.2.1 Cancer Targeting with Polymeric Drugs

Polymeric drugs (also referred to as macromolecular drugs) are a diverse group of drugs including polymer-conjugated drugs, polymeric micelles, liposomal drugs, and solid-phase depot formulations of various agents. Water-soluble macromolecular drugs have high molecular weights, more than 40 kDa, which enables them to overcome renal excretion. Consequently, this group of drugs can attain prolonged plasma or local half-lives. The prolonged circulating time of these macromolecules

Table 15.2 Innovative methods of drug delivery in cancer

Cancer targeting with polymeric drugs
Polyamine conjugates as anticancer agents
Polyethylene glycol (PEG) technology
Monoclonal antibody (MAb)-based anticancer drug delivery
Bacteria as drug delivery systems for anticancer drugs
Cell-based drug delivery
Microparticles as therapeutic delivery systems in cancer
Nanobiotechnology-based drug delivery in cancer
Release of drugs from conjugates by physical agents
Release of drugs by ultrasound
Release of drugs by laser

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enables them to utilize the vascular abnormalities of solid tumor tissues, a phenomenon called the enhanced permeability and retention (EPR) effect. EPR effect is attributed to anatomical and pathological alterations such as increased vascular density due to angiogenesis, impaired lymphatic recovery, and lack of smooth muscle cells in solid tumor vessels. As a result, the macromolecular drug concentration in tumor tissues far exceeds that of blood plasma, usually by five- to tenfold, which would be difficult to achieve with low molecular weight drugs. The EPR effect facilitates extravasation of polymeric drugs more selectively at tumor tissues, and this selective targeting to solid tumor tissues may lead to superior therapeutic benefits with fewer systemic adverse effects. This contrasts with conventional low molecular weight drugs, where intratumor concentration diminishes rapidly in parallel with plasma concentration. At the present time, several polymeric drugs have been approved by regulatory agencies. These include zinostatin stimalamer (copolymer styrene maleic acid-conjugated neocarzinostatin or SMANCS) and polyethylene glycol (PEG)-conjugated interferon-α-2a.

15.2.1.1 Linking Anticancer Drugs to Polyglutamate

This technology links anticancer drugs to proprietary polymers, such as polyglutamate (PG). PG, under development by Cell Therapeutics Inc., is a biodegradable polymer of glutamic acid—a naturally occurring amino acid. It is built by linking glutamic acid molecules together to an optimal size.

Polyglutamate drug delivery technology is a new way to deliver cancer drugs more selectively to tumor tissue with the goal of reducing the toxic side effects and improving the anticancer activity of existing chemotherapy agents. Polymer technology takes advantage of a well-described difference between tumor blood vessels and blood vessels in normal tissues. The blood vessels in tumor tissues are more porous than those in normal tissues, and they are therefore more permeable to large molecules, such as polymers, that are within a specific size range. As the polymer, carrying its tumor-killing drug, circulates in the bloodstream and passes through the tumor blood vessels, it becomes trapped in the tumor tissue allowing a significantly

greater percentage of the anticancer drug to accumulate in tumor tissue compared to normal tissue. The toxicity of the chemotherapy drug to normal tissues also may be reduced because the drug appears to be inactive as long as it is bound to the polymer. Once the polymer backbone is digested in the tumor, the cancer-killing drug is released directly into the cancer tissues.

Based on preclinical animal studies and clinical trial data, polymer–chemotherapy drug conjugates have a number of benefits over existing chemotherapy drugs:

- More drug reaches the tumor
- Increased efficacy using the same amount of active drug
- Ability to use higher doses of the active drug
- Less toxicity at the same or higher doses of active drug
- Broader applicability due to differentiated tumor uptake mechanism
- Potential to overcome resistance to the underlying chemotherapy drug

Paclitaxel poliglumex (Cell Therapeutics' Xyotax™) links paclitaxel, the active ingredient in Taxol®, to PG resulting in a new chemical entity, designed to selectively deliver higher and potentially more effective levels of active chemotherapeutics to tumors. Based on preclinical studies, it appears that Xyotax™ is preferentially trapped in the tumor blood vessels allowing significantly more of the dose of chemotherapy to localize in the tumor. Because more of the chemotherapy is targeted to the tumor and the levels of chemotherapy delivered to normal tissue are reduced, Xyotax™ may be potentially more effective and have less severe side effects than currently available chemotherapeutics. Phase III clinical trials in lung cancer have been completed. Gender may affect the action of Xyotax as estrogen seems to be involved in breaking down the polyglutamate polymer and releasing paclitaxel to cancer cells. Estrogen may also distribute Xyotax to cells with estrogen receptors. On the other hand, the hormone may speed the release or metabolism of the drug.

15.2.1.2 Improving Delivery of Protein–Polymer Anticancer Drugs

Protein-based drugs are an increasingly important new class of anticancer drugs, e.g., antibodies like Herceptin. Unmodified proteins that are injected into the blood are quickly recognized by the body and broken down or cleared by the body's defense system, which limits their effectiveness as drugs. To overcome this problem, PEG has been attached to the protein in order to protect it, but this approach is only 10–20 % effective. Moreover, two large molecules are attached by a small chemical link and, because these linkages often occur at many different sites on the protein, the final product is poorly defined. Bioengineers at Duke University (Durham, NC) developed the new approach (Gao et al. 2009). Instead of combining two large molecules, they grew the polymer out from the protein itself. They used myoglobin, and instead of creating a chemical bond between myoglobin and the polymer, they chose a specific spot on the protein, known as the N-terminus, and then grew the polymer from that specific location. Since every protein has an N-terminus, this method

should be broadly applicable. This approach increased the efficiency of the protein delivery by more than 70 % and greatly extending the amount of time it remained active in a living model. Other advantages of the new approach are as follows: (1) the product obtained is well defined as the polymer is grown from a single, unique site on the protein, and (2) the polymer used is better than PEG in extending circulation of the protein in the body. Current protein–polymer drugs will be tested to determine if the new technique can improve their effectiveness.

15.2.2 Macromolecules as Delivery Systems for Taxanes

Macromolecule conjugates have been widely investigated along other taxane delivery systems. Natural and synthetic polymers, proteins, and polysaccharides have been covalently coupled with taxanes; immunoconjugates have also been developed for targeted delivery. The choice of macromolecule, the spacer, and the chemistry of the linkage with taxane, as well as other cytotoxic drugs, are key factors for obtaining effective conjugates with higher activity than that of the free drug and reducing side effects (Dosio et al. 2011). Critical evaluation of the different approaches may help in understanding and comparing the results and may elucidate the role of individual components. Taxane covalently bound to macromolecules shows advanced properties, and although only one compound is in advanced clinical trials, this area deserves attention and seems a promising route to achieve effective new anticancer compounds.

15.2.3 Polyamine Conjugates as Anticancer Agents

A polyamine conjugate is a special polyamine derivative composed of polyamine vectors appended directly or by a linker to a cargo with specific biological functions. Polyamine conjugates are promising as anticancer agents. Design of polyamine conjugates follows a rational mechanism-based strategy with a focus on the molecular recognition of polyamine transport system (Xie et al. 2010). Multiple functions of polyamine moieties in objective conjugates provide broad development opportunities for more effective anticancer agents.

15.2.4 Bacteria as Vehicles for Delivery of Anticancer Drugs

There are several approaches—bactofection, bacterial ghosts, and bacterially mediated protein and RNAi delivery—in which modified bacteria can be used for delivery of anticancer therapeutics. Live bacterial vectors may be useful tools for the

development of novel cancer therapies that can be added to the repertoire of existing drugs. Several bacterial strains effectively colonize solid tumors and act as antitumor therapeutics. The naturally occurring tumor-colonizing characteristics of bacterial species such as *Salmonella* sp., *Clostridium* sp., and *E. coli* can be further modified by genetic manipulations, making these bacterial systems excellent vehicles for the production and targeted delivery of therapeutic molecules into cancer cells (Gardlik and Fruehauf 2010).

Bacterial ghosts are empty non-denatured envelopes derived from gram-negative bacteria with fully intact surface structures for specific attachment to mammalian cells. The bacterial ghost system represents a platform technology for antigen, nucleic acid, and drug delivery. Bacterial ghosts have significant advantages over other engineered biological delivery particles, because of their intrinsic cellular and tissue tropic abilities, ease of production, and the fact that they can be stored and processed without the need for refrigeration. These particles have applications for the vaccination and treatment of tumors. Their ease of manufacture, storage without refrigeration, and safety profile make them attractive agents for drug delivery.

15.2.5 Microparticles as Therapeutic Delivery Systems in Cancer

Microparticles, also referred to as microspheres and prepared from cross-linked proteins, have been used as biodegradable drug carriers. The rate of release of small drug molecules from protein microspheres is relatively rapid, although various strategies such as complexing the drug with macromolecules can be adopted to overcome this problem. Polysaccharides (e.g., starch) and a wide range of synthetic polymers have been used to manufacture microspheres. Microcapsules differ from microspheres in having a barrier membrane surrounding a solid or liquid core, which is an advantage in case of peptides and proteins; their applications in cancer therapy are shown in Table 15.3.

15.2.5.1 Subcutaneous Injection of Polymer Carrying Anticancer Drugs

Eligard® (Sanofi), an approved polymer-based drug delivery system, was proven safe and effective for systemic drug delivery during human clinical studies for prostate cancer. It is injected into the body subcutaneously as a liquid, where it solidifies and delivers a dose of leuprolide acetate at a controlled rate over the specified therapeutic period of time. A 6-month formulation of Eligard® 45 mg has been approved by the FDA for the palliative treatment of advanced prostate cancer. Unlike microcapsules or microspheres, which are usually injected intramuscularly, it can also be administered subcutaneously, a method that is simpler and less painful, and can be easily retrieved if treatment needs to be terminated. It can be administered with a

Table 15.3 Microparticles as therapeutic delivery systems in cancer

Microspherical systems loaded with the cytotoxic drugs
5-Fluorouracil
Cisplatin
Doxorubicin
Mitomycin C
Paclitaxel encapsulated in polilactofate microspheres (Paclimer)
Tumor embolization with radioactive microparticles for imaging and radiotherapy
Anticancer drug bound to carbon microparticles
Tumor targeting by magnet-guided microparticles
Release of drugs from biSphere by ultrasound
Chemoembolization
Encapsulation of cancer biopharmaceuticals in microparticles
IL-1α in PLGA
IFN-γ and IFN-α 2b in PLGA
IL-2 in chitosan
GM-CSF in gelatin
Injection of preparation containing microparticle-based anticancer drugs
Subcutaneous injection of microspheres carrying anticancer drugs
Intravenous injection
Microparticle-based biological therapies
DNA vaccines
Antisense oligonucleotides
Gene therapy

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standard needle and syringe, and unlike many implants now used for long-acting drug delivery, it is bioabsorbable and does not require surgical removal.

15.2.5.2 Intravascular Delivery Systems Using Microparticles

Polymer conjugates have been used for systemic delivery of chemotherapeutic agents. The following forms are used:

- Soluble polymer complexes, which deliver the drug in high concentrations to tumor cells and avoid exposure of healthy cells to the drug.
- Nanoparticles of polycyanoacrylate, which tend to accumulate in tumors. Drugs used for nanoparticle delivery are mostly anticancer agents such as actinomycin D, 5-fluouracil, doxorubicin, and methotrexate.
- Insoluble polymer complexes, which are used both parenterally and as implants. They serve as extracellular drug depots and slowly and continuously release the drugs.

The advantages of polymer-based intravenous systemic delivery of chemotherapeutics are as follows:

- They enhance tumor cell targeting.
- They improve stability of anticancer agents.

- They enable high concentrations of chemotherapeutics in the tumors while reducing systemic toxicity.
- Polymer carriers may lower or overcome the multidrug-resistance barrier to chemotherapy.

15.2.5.3 Tumor Embolization with Drug-Eluting Beads

DC Bead™ (Biocompatibles International plc) is a drug delivery embolization system for PRECISION TACE (transarterial chemoembolization). DC Bead™ is specifically designed to be loaded with doxorubicin for the purpose of (1) embolization of vessels supplying malignant hypervascularized tumors and (2) delivery of a local, controlled, and sustained dose of doxorubicin to the tumors (Lewis and Holden 2011). This product is finding increasing use in the treatment of patients with both primary and secondary liver cancers.

Biocompatibles is now combining its expertise in drug delivery with the N-fil Technology platform to advance the potential for embolization therapy in the treatment of cancer by drug-eluting beads. Currently embolic products are combined with chemotherapy in a procedure called chemoembolization. The drug-eluting bead under development aims to enhance the effectiveness of this treatment while simplifying the procedure for the interventional radiologist. The company is currently applying its drug-elution technology to the development of drug-eluting embolic beads for use in embolization therapy to treat liver cancer.

15.2.5.4 Tumor Embolization with Radioactive Microparticles

Several attempts have been made during the past several years to destroy tumors by injected microparticles into arterial supply of tumor. So far success has been achieved only in treatment of benign tumors. BioSphere Medical's Embospheres, approved by the FDA, are precisely calibrated, spherical, hydrophilic, and microporous beads made of an acrylic copolymer (trisacryl) which is then cross-linked with gelatin. This proprietary and patented design allows a more complete and targeted occlusion of the blood vessels feeding a hypervascularized tumor or arteriovenous malformation. They are used for embolization of benign uterine fibroids as alternative to surgery. BioSphere Medical is now developing a microsphere formulation that is capable of transporting radiotherapy via the vascular system to cancerous liver tumors. Simultaneous diagnostic imaging will allow clinicians to measure precisely the radiation dose to the tumor, a feature lacking in currently available radioactive microspheres.

15.2.5.5 Release of Drugs from biSphere by Ultrasound

A biSphere™ (Point Biomedical) is a shell within a shell. The inner shell, formed from biodegradable polymers, provides physical structure and controls acoustic

response, while the outer layer functions as the biological interface and can provide a scaffold for site-specific targeting ligands. In drug delivery applications, a drug-loaded version of the biSphere is essentially directed to release the active agent within any given volume of tissue that can be addressed with ultrasound. The biSpheres would be administered intravenously and freely circulate throughout the body, while the drug encapsulated within would remain biologically unavailable. The drug would only be released when the biSpheres become flooded when passing through an externally directed ultrasound field. The rapid dissolution of the internal gas upon flooding of the biSpheres can be detected providing feedback as to the amount of drug being released at the site. This technology enables the biodistribution of the drug to be controlled and high concentrations of drug to be locally delivered and maintained. The use of biSpheres to transport agents to specific sites within the body is expected to substantially increase local efficacy while decreasing systemic side effects or adverse reactions. The biSpheres may also serve to protect labile agents from metabolism or degradation. The noninvasive release of a protected, encapsulated agent can be controlled by ultrasound imaging to a depth of 20–30 cm from the skin surface. Point Biomedical is currently researching applications of the technology for use in treating heart disease, cancer, and other tissue- or lesion-specific conditions.

15.2.5.6 Release of Drugs from Microcapsules by Laser

For targeted delivery in cancer, anticancer drugs are enclosed in microcapsules that are channeled into cancer cells and are then exposed to laser light which cracks the polymer shells by heating them up, releasing the contents (Skirtach et al. 2006). The polymer capsules are only a few micrometers in diameter, and the walls of the capsules are built from a number of layers of charged polymers, alternating positive and negative. This is an established method of producing transport containers for medicines, cosmetics, or nutrients, which can also pass through cell membranes. Metal nanoparticles, incorporated in the polymers composing the walls of the capsules, are particularly good at absorbing the laser light and transmitting the heat further into their surroundings, heating up the walls. The heat breaks the bonds between the polymers and the shell and the capsules eventually open.

15.2.5.7 Chemoembolization

Microspheres can be used for chemoembolization of tumors in which the vasculature is blocked while the anticancer agent is released from the trapped microparticles. The rate at which the drug is released from the microparticles is dependent on three main factors:

- Solubility of the encapsulated drug and diffusion
- Rate of particle biodegradation
- Complex formation between the drug and the particle matrix, leading to immobilization

15.3 Polyethylene Glycol Technology

PEG is a polyether compound with many applications including pharmaceutical manufacturing and drug delivery technologies. PEGylation is the act of covalently coupling a PEG structure to another larger molecule, for example, a therapeutic protein, which is then referred to as a PEGylated protein. PEG is used as an excipient in many pharmaceutical products. Gene therapy vectors (such as viruses) can be coated with PEG to shield them from inactivation by the immune system. PEG is a component of stable nucleic acid lipid particles (SNALPs) used to package siRNA for use in vivo (see Chap. 12).

Enzon's PEG chemotherapy combinations and Nektar PEGylation will be described as examples.

15.3.1 Enzon's PEG Technology

Enzon is applying PEG technology to small molecules. Like proteins, many small molecules of potentially significant therapeutic value possess undesired pharmacologic characteristics such as poor solubility, limited half-life, and propensity to induce an immunologic response. This PEGylation technology employs proprietary chemical linkers designed to either release the native molecule at a controlled rate or provide permanent linkage that will maximize inherent activity of the native molecule. In some cases, PEGylation can render a compound therapeutically effective, whereas the unmodified form had only limited clinical utility. A number of marketed biologic products utilize this novel PEGylation platform.

The attachment of PEG to small molecules not only disguises the molecule, thereby lowering potential immunogenicity and extending its circulatory life, but also greatly increases the solubility of these compounds. PEG is attached to small molecules by means of a covalent bond that is designed to temporarily inactivate the compound and then deteriorate over time, releasing the compound in the proximity of targeted tissue. By inactivating and then reactivating the compound in the body, a prodrug version of such compounds is created. These attributes may significantly enhance the therapeutic value of new chemicals, drugs already marketed, and off-patent drugs with otherwise limited utility. This technology can be applied to a wide range of small molecules such as cancer chemotherapy agents.

One of the examples of application of PEG technology is the chemotherapy agent L-asparaginase, which has been an important part of acute lymphoblastic leukemia therapy for over 30 years. Two of the main disadvantages of the drug are (1) the need for frequent intramuscular injection and (2) a very high rate of allergic reactions. Because of this, L-asparaginase seemed like an ideal target for pegylation and PEG-L-asparaginase was developed in the 1970s and 1980s. The drug has undergone extensive testing and appears to retain its antileukemic effectiveness while allowing less frequent administration than the native compound. Oncaspar (Enzon), a

PEG-modified version of the L-asparaginase, is now an approved chemotherapeutic agent for acute lymphoblastic leukemia.

PEG-SN38 (EZN-2208), a PEGylated conjugate of SN38, offers therapeutic advantages over unmodified SN38 and existing therapies. The PEGylated version allows parenteral delivery, increased solubility, higher exposure, more profound DNA damage, inhibition of angiogenesis, and longer apparent half-life of SN38. It is in phase II clinical trials for metastatic breast cancer and metastatic CRC.

15.3.2 Nektar PEGylation

Nektar PEGylation technology (Nektar Therapeutics) can enhance the properties of therapeutic agents by increasing drug circulation time in the bloodstream, decreasing immunogenicity and dosing frequency, increasing bioavailability, and improving drug solubility and stability. It can also be used to modify pharmaceutical agents to preferentially target certain systems within the body. It is a technique in which PEG polymers are attached to therapeutic agents, and it is applicable to most major drug classes, including proteins, peptides, antibody fragments, small molecules, and other drugs. Nektar has advanced PEG technology platform to apply to a broader range of molecules and to overcome the limitations of the first generation of the technology platform.

Nektar is developing etirinotecan pegol (NKTR-102), a PEGylated form of irinotecan invented by using its leading small-molecule PEGylation technology platform. Irinotecan is used for the treatment of solid tumors. By applying Nektar's small-molecule PEGylation technology to irinotecan, NKTR-102 may prove to be a more powerful and tolerable anticancer agent. In preclinical studies on tumor-bearing mice, NKTR-102 resulted in significantly reduced tumor growth compared to irinotecan in colon, lung, and breast tumors. These studies indicate that Nektar's small-molecule PEGylation technology may enable NKTR-102 to have prolonged systemic exposure following intravenous administration. Furthermore, preclinical studies in mice indicate that NKTR-102 was well tolerated with significant reduction of neutropenia and diarrhea, two debilitating side effects of non-PEGylated irinotecan. It is in phase III trials for metastatic breast cancer.

15.3.3 PEG Intron

PEG Intron (PEGylated IFN-α-2b, Merck & Co.) is approved for the treatment of hepatitis C. It is in phase III clinical trials for malignant melanoma and preclinical investigations for the treatment of solid tumors. PEG Intron has demonstrated delayed clearance and increased area under the curve compared with native IFN-α-2b. Studies in patients with chronic hepatitis C infection and malignancies have

demonstrated both biological and clinical activity of PEG Intron and have provided empiric data to compare the pharmacokinetics and pharmacodynamics of PEG Intron and IFN-α-2b. PEG Intron at doses up to 6 μg/kg per week was well tolerated and demonstrated clinical activity in patients with CML and solid tumors, including metastatic melanoma and renal cell carcinoma. Dose intensification can be achieved safely in patients with CML and solid tumors using PEG Intron, which could improve efficacy. These results provide useful dosing guidelines to clinicians investigating the antitumor activity of PEG Intron in patients with malignancies. However, these results provide a sound rationale for further investigation of PEG Intron.

15.4 Single-Chain Antibody-Binding Protein Technology

Single-chain antibody-binding (SCA) proteins, like MAbs, deliver therapeutic proteins to targeted disease sites. The advantages over MAbs are as follows:

- SCA proteins are easier to produce and do not need to go through the humanization process.
- SCAs penetrate the tumor much better because their molecular weight is only a fraction of that of the usual antibodies.
- Immunogenicity is reduced because protein that is not required for antigen binding is not included.
- Flexibility to tailor half-life via PEG technology.
- More cost-effective scale-up for manufacturing when compared with MAbs.
- Better delivery opportunities offering potential for non-parenteral delivery.

Enzon is developing SCA technology for delivery of cancer therapeutics. Using an ^{123}iodine-labeled SCA selected from a combinatorial library, clinical evidence of efficient tumor targeting in patients with CEA-producing cancer has been demonstrated.

15.5 Vesicular Systems for Drug Delivery in Cancer

Biological membranes form the ubiquitous delimiting structures that surround and compartmentalize all cells and organelles. The bilayer arrangement of lipids is perhaps the only organizational feature that is common to all biological membranes. Lipid vesicles, one of the many experimental models of biomembranes, can play a major role in modeling biological membranes and in the transport and targeting of active agents. Although developed for basic research, many technological innovations have arisen from the applications of these models. Lipid vesicles have evolved successfully, as vehicles for controlled delivery and can be used for anticancer drugs.

Vesicular drug delivery systems can overcome limited permeation of drugs into cells. Encapsulation of a drug in vesicular structures can be predicted to prolong the existence of the drug in systemic circulation and may reduce toxicity if selective uptake can be achieved. Vesicular drug delivery reduces the cost of therapy by improved bioavailability of medication, especially in case of poorly soluble drugs. They can incorporate both hydrophilic and lipophilic drugs. Vesicular drug delivery systems delay drug elimination of rapidly metabolizable drugs and function as sustained release systems. This system solves the problems of drug insolubility, instability, and rapid degradation. Consequently, a number of vesicular delivery systems such as liposomes, niosomes, and pharmacosomes have been developed.

15.6 Liposomes for Anticancer Drug Delivery

Liposomes are closed lipid spheres and are well established as drug carriers. Liposomes may circulate in the bloodstream for extended periods, as compared to the same drug in a non-liposomal form. This may result in an extended treatment effect and a simplified dosing regimen for physicians and patients. In some cases, liposomal drugs have been shown to accumulate at the site of a tumor or infection delivering higher concentrations of the drug to a disease target. The liposome carrier is believed to play a role in reducing the harmful effects of certain drugs on healthy tissues, thereby offering the potential for an improved safety profile for certain drugs. The largest application of liposomes is in cancer therapy because encapsulation with liposome increases the efficacy of anticancer drugs. The advantages of encapsulating anticancer drugs in liposomes are as follows:

- They target drug delivery to tumor tissue rather than to normal tissue. Liposomes containing 5-FU, for example, target primary hepatocellular carcinoma.
- They prolong the duration of drug availability in the tumor.
- They reduce the toxicity of anticancer agents.
- Encapsulating the anticancer drug doxorubicin in liposome in combination with PSC-833 enhances the effect against multidrug resistance without adding the risk of toxicity.

The limitations of conventional liposomal therapy for cancer are as follows:

- The difficulty of finding a suitable lipid/drug combination and reproducing the results of in vitro studies in vivo.
- The rapid disintegration that liposomes undergo in the intestine when given orally.
- The oxidation by macrophages of the reticuloendothelial system that occurs within seconds following intravenous injection. The result is a rupture of the liposome and premature release of the drug.
- The tagging of the liposome by macrophages for removal by the reticuloendothelial system, an occurrence that can take place within minutes to a few hours. Removal of the drug from circulation within such a short time provides no opportunity for reaching the cancer site.

15.6.1 Antibody-Targeted Liposomes for Cancer Therapy

Targeted liposomes have been used in diagnosis to deliver contrast agents and radionuclides for magnetic resonance and nuclear medicine imaging. They have been used in gene therapy to deliver a variety of gene expression modifiers, including plasmids, antisense oligonucleotides, and shRNAs. Targeted liposomes provide a delivery advantage over untargeted liposomes not only because of increased localization to tumor sites but also because of increased interaction with the target cell population once localized to the tumor site, which can take on the form of fusion with the cellular membrane or internalization by endocytosis. Spatial distribution of targeted liposomes within a solid tumor may be less uniform than that of untargeted liposomes, but this has not been documented so far. Most of the reported studies of antibody-targeted liposomes are in the area of chemotherapy delivery. Their use in radionuclide and chemo- and radiosensitizer delivery is just emerging. Multifunctional liposomes containing "layered functionalities" could potentially be the future direction in targeted liposome-based therapy (Sofou and Sgouros 2008).

15.6.2 Stealth Liposomes

Stealth® technology (Janssen) is composed of lipid nanoparticles that incorporate a PEG coating. This coating helps evade the immune system and enables Stealth technology to provide the precise delivery of drugs to disease-specific areas of the body. Doxil®, the first marketed product to incorporate Stealth® technology, is an anticancer drug for the treatment of refractory ovarian cancer and AIDS-related Kaposi's sarcoma. The advantages of this technology are as follows:

- Tissue targeting. Stealth technology targets the leaky vasculature associated with tumor and diseased tissues, thereby increasing drug concentration at these sites.
- Reduced toxicity. Stealth technology can alter the biodistribution of many therapeutic agents, by directing them away from organs responsible for side effects.
- Improved drug solubility. Many insoluble and poorly soluble drugs can be incorporate into Stealth liposomes, resulting in an improved solubility profile.
- Intracellular drug delivery. Stealth liposomes bearing ligands can target receptors expressed on diseased cells. This ligand binding promotes efficient drug uptake into cells and enhances efficacy.

The most advanced drug formulation that uses this approach is a doxorubicin liposome to which an Her2-targeting antibody has been attached. Pegylated liposomal formulation of doxorubicin has been evaluated in patients with metastatic breast cancer previously treated with conventional anthracyclines (Al-Batran et al. 2006). All patients were previously treated with chemotherapy, and 30 % of patients had prior chemotherapies for metastatic disease. Pegylated liposomal doxorubicin was associated with an evident clinical benefit in anthracycline-pretreated patients with metastatic breast cancer.

15.6.3 Boron-Containing Liposomes

One study has investigated HER-2-targeted boron-containing liposomes as a potential drug delivery vehicle for boron neutron capture therapy (BNCT). Trastuzumab was conjugated to the distal end of PEG-DSPE-NHS in micelles, and the trastuzumab-PEG-DSPE was then transferred to preformed liposomes, either empty or loaded with water-soluble boronated acridine (WSA), using the micelle transfer method (Wei et al. 2003). The final conjugates were referred to as trastuzumab–liposome and trastuzumab–liposome–WSA. The conjugates showed specific binding to the HER-2 receptors of SK-BR-3 cells. High cellular uptake and internalization of the conjugates were seen. WSA was distributed mainly in the cytoplasm and was shown to have long cellular retention. The conjugate trastuzumab–liposome–WSA could be considered as a potent drug delivery system for BNCT.

15.6.4 DepoFoam Technology

The DepoFoam technology (Pacira Pharmaceuticals Inc.) involves an aggregation of liposomes that are 10–30 μm in size and is specifically designed to provide a sustained release of the drug for up to 1 month. The technology has already been proven and is amenable to further development, as demonstrated by the market introduction of DepoCyt, used in neoplastic meningitis by intrathecal delivery. The formulation, which is administered once every 2 weeks, has overcome the limitations of current therapies for such disease (i.e., two to three doses per week by means of lumbar punctures and rapid clearance of conventional formulations that results in inadequate distribution in the CSF). This has significantly improved the pharmacokinetic profile of the active drug substance (its half-life in the ventricular CSF is 141 h vs. 3.4 h when given in conventional formulations) and its clinical efficacy (higher response rate, 72 % vs. 18 %, and an increase of 60 % in the median time to neurological progression).

15.6.5 Hyperthermia and Liposomal Drug Delivery

Hyperthermia and liposomal drug delivery have each been used to treat cancer over the last 2 decades. More recently, the two therapies have been used together in an attempt to exploit their mutual interactions against cancer. A review of the literature has shown that hyperthermia, in combination with liposomal drugs, has an enhanced therapeutic effect compared with either treatment modality alone or hyperthermia and a drug without liposomal formulation.

A heat-sensitive liposomal drug delivery system was tested on tumors grown subcutaneously in mice using Colon-26 (CT-26) cultured cells and Lucifer yellow iodoacetamide (LY) as a fluorescence marker (Wells et al. 2003). LY released in tumors

was determined from fluorescence intensity. Tumors receiving heat-sensitive liposomes plus heat treatment showed 2.5-fold greater fluorescence than all other tumors, which were at the background level. This study demonstrates the possible use of poloxamer-containing liposomes as a heat-sensitive drug delivery system in vivo.

Celsion has developed ThermoDox™ (thermally sensitive liposomal doxorubicin), a heat-sensitive liposome microcarrier encapsulating the anticancer drug doxorubicin, which is administered intravenously. Celsion's precise thermoarray system, called "adaptive phased-array thermotherapy," is used to focus heat on the tumor site to trigger release of the drug contents of the microcarriers within a few minutes. The aim of this approach is to achieve increased concentrations of the anticancer drug within the tumor. Preclinical studies have shown that this technique increases the intratumor drug concentration about 50-fold at a temperature of 42 °C. A phase III trial was conducted in patients with nonresectable liver cancer. Future work needs to focus on optimizing thermosensitive liposomes and understanding the effect of thermal dose on liposomal drug delivery. Though not yet approved for use in clinical practice, this combination therapy seems to hold great promise toward improving current cancer therapeutic regimens.

15.6.6 Liposomal Doxorubicin Formulation with **N-***Octanoyl-glucosylceramide*

The anticancer agent doxorubicin is in certain cases administered as a long-circulating liposomal formulation. Due to angiogenesis-related structural abnormalities in the endothelial lining of many neoplasms, these complexes tend to extravasate and accumulate in the tumor stroma. However, delivery of doxorubicin is still not optimal since liposomes are not taken up directly by tumor cells. Instead, doxorubicin is gradually released into the interstitial space, and the subsequent uptake by surrounding cells is a limiting step in the delivery process. Several formulations have sought to improve the delivery of liposomal doxorubicin. Plasma membrane-inserted short-chain sphingomyelin facilitates the cellular uptake of free doxorubicin. *N*-octanoyl-glucosylceramide, a short-chain glycosphingolipid, when coformulated with Caelyx (Merck's commercial liposomal doxorubicin), leads to higher (up to fourfold) cellular doxorubicin accumulation and anticancer effect, compared with control doxorubicin liposomes (Veldman et al. 2005). *N-octanoyl-glucosylceramide* enrichment might thus represent a major improvement of conventional liposomal doxorubicin formulations.

15.6.7 Liposome–Nucleic Acid Complexes for Anticancer Drug Delivery

Scientists at the NCI have created liposome–nucleic acid complexes, which have extended half-life in the circulation, avoid endosomes, penetrate through tight

barriers in several organs, and have broad biodistribution. They can efficiently encapsulate various sizes of nucleic acids or other molecules including drugs, are fusogenic with cell membranes, and are targetable to specific organs and cell types. These can be size-fractionated to produce a totally homogenous population of complexes prior to injection, are nontoxic as well as non-immunogenic, and can be repeatedly administered. Liquid suspensions and freeze-dried formulations are stable. These complexes have been injected into mice, rats, rabbits, pigs, and nonhuman primates. They are being administered intravenously to patients in clinical trials for treatment of lung cancer and will be used in upcoming trials to treat breast, pancreatic, and head and neck cancers. The improved liposome complexes could increase the efficacy of treatments for cancer.

15.6.8 Non-PEGylated Liposomal Doxorubicin

Non-PEGylated liposomal doxorubicin is a second-generation product and differs from other formulations as the active substance is placed in the wall of the liposome and not inside and contains a small percentage of chromane, a molecule with antioxidant properties that stabilizes the liposome. This reduces toxicity and increases distribution as well as availability of doxorubicin. This preparation of liposomal doxorubicin, as a single agent, has proven superior to other currently used drugs for treatment and overall survival of soft tissue sarcomas. A retrospective study has evaluated the experience obtained with non-PEGylated liposomal doxorubicin as first-line therapy in patients with metastatic breast cancer (Livi et al. 2009). The overall response rate was 71 %. The median progression-free survival was 8 months in patients receiving non-PEGylated liposomal doxorubicin plus cyclophosphamide and 13.8 months in those receiving non-PEGylated liposomal doxorubicin plus docetaxel. These results support the use of non-PEGylated liposomal doxorubicin as an alternative to conventional doxorubicin formulations in combination regimens for the first-line therapy of metastatic breast cancer.

15.6.9 Tumor-Selective Targeted Drug Delivery via Folate–PEG Liposomes

The molecular target of this approach is the folate receptor, which is overexpressed in many types of human cancers. Folic acid enters cells either through a carrier protein, termed the reduced folate carrier, or via receptor-mediated endocytosis facilitated by the folate receptor. Because folate–drug conjugates are not substrates of the former, they penetrate cells exclusively via folate receptor-mediated endocytosis. In vitro studies have shown that tumor cells take up liposomes conjugated to folate via receptor-mediated endocytosis. Preclinical research in animal models is in progress to determine the tumor selectivity of folate-targeted liposomes entrapping doxorubicin in vivo. PEG-liposomes are also suitable for cancer gene therapy.

15.6.10 Ultrasound-Mediated Anticancer Drug Release from Liposomes

To enhance tumor uptake and selectivity of drugs, liposomally encapsulated microbubbles with drugs or temperature-sensitive liposomes with therapeutics have been suggested as new drug delivery vehicles, in combination with ultrasound or hyperthermia, respectively. One release model involves targeting, real-time monitoring, and magnetic resonance imaging (MRI), within a multimodal treatment regime, thus enhancing synergism (Myhr 2007). A digital tumor model can facilitate an optimal treatment procedure. The system integrates a diagnostic unit with a therapeutic ultrasonic transmitting component, together with a central processing unit, encompassing algorithms for data processing and visualization. Actual drug uptake is based on passive accumulation of drug carriers. Selective drug release of, e.g., cytostatic drugs is achieved by ultrasound induced cavitation confined to the tumor. Further research related to optimal timing, combinations of responses between liposomally encapsulated drug dosage, ultrasound exposure, hyperthermia, pO_2 response time, ionizing radiation fractionation, and treatment time has been proposed (Myhr 2008a, b).

15.7 Pharmacosomes for Controlled Anticancer Drug Delivery

Pharmacosomes are amphiphilic phospholipid complexes of drugs bearing active hydrogen that bind to phospholipids. The prodrug conjoins hydrophilic and lipophilic properties, and therefore acquires amphiphilic characters, and similar to other vesicle forming components, reduces interfacial tension, and at higher concentrations exhibits mesomorphic behavior. These are defined as colloidal dispersions of drugs covalently bound to lipids and may exist as ultrafine vesicular, micellar, or hexagonal aggregates, depending on the chemical structure of drug–lipid complex. Many limitations of classical vesicular drug delivery systems, such as problems of drug incorporation, leakage from the carrier, or insufficient shelf life, can be avoided by the pharmacosome approach. Some features of pharmacosomes are (Biju et al. 2006):

- Entrapment efficiency is not only high but predetermined, because drug itself conjugates with lipids to forms vesicles.
- Unlike liposomes, there is no need of following the tedious, time-consuming step for removing the free, unentrapped drug from the formulation.
- Since the drug is covalently linked, loss due to leakage of drug does not take place. However, loss may occur by hydrolysis.
- Encaptured volume and drug–bilayer interactions do not influence entrapment efficiency, in case of pharmacosome. These factors, on the other hand, have great influence on entrapment efficiency in case of liposomes.

- The lipid composition in liposomes decides its membrane fluidity, which in turn influences the rate of drug release and physical stability of the system. However, in pharmacosomes, membrane fluidity depends upon the phase transition temperature of the drug–lipid complex, but it does not affect release rate since the drug is covalently bound.
- Due to their amphiphilic behavior, such systems enable multiple transfer of drugs through the lipophilic membrane system or tissue or cellular walls piggyback endocytosis and exocytosis.
- Following absorption, their degradation velocity into active drug molecule depends to a great extent on the size and functional groups of drug molecule, the chain length of the lipids, and the spacer. These can be varied for optimized in vivo pharmacokinetics.
- They can be administered orally or topically as well as extra- or intravascularly.

Pharmacosomes improve biopharmaceutical properties of the drug, resulting in improved bioavailability. Pharmacosomes have been prepared for various antineoplastic drugs. Developing the pharmacosomes of the drugs has been found to improve the absorption and minimize the gastrointestinal toxicity.

15.8 Albumin-Based Drug Carriers

A-based drug carrier systems have been developed to improve the passive tumor-targeting properties of anticancer drugs. Because of impaired lymphatic drainage, macromolecules like albumin accumulate to a considerable extent in tumor tissues. After endocytosis by tumor cells, albumin is catabolized, and resulting amino acids are used for tumor protein synthesis. At this stage, the attached anticancer drugs are also released and can exert their action on cancer cells.

Albumins of different species—mostly bovine, human, or rat—have been used as carrier proteins. Albumin–drug conjugates have the same favorable tumor-targeting properties as albumin (i.e., high tumor uptake rate, low liver uptake, and long biological half-life). Multiple injections of human serum albumin conjugated with methotrexate are tolerated without signs of anaphylaxis. Phase I testing of albumin–methotrexate conjugate showed a favorable toxicological profile, enabled outpatient treatment, and maintained a high quality-of-life status for all patients. However, there has been no commercial development of this technology.

15.9 Radioactive Materials for Diagnosis and Targeted Therapy of Cancer

Nuclear medicine has used radiotherapy for the treatment of cancer and radioactive materials are used for diagnosis as well. Both functions can be combined.

15.9.1 Pretargeted Radioimmunotherapy of Cancer

Pretargeted radioimmunotherapy (PRIT) is designed to enhance the directed delivery of radionuclides to malignant cells. The potential therapeutic advantage of anti-CD45 PRIT was evaluated through a series of studies in nonhuman primates (Green et al. 2009). Anti-CD45 PRIT demonstrated a significant improvement in target-to-normal organ ratios of absorbed radiation compared with directly radiolabeled bivalent antibody (conventional radioimmunotherapy). PRIT generated superior target-to-normal organ ratios in the blood, lung, and liver compared with the conventional RIT. The anti-CD45 streptavidin fusion protein (FP) demonstrated superior retention in target tissues relative to comparable directly radiolabeled bivalent anti-CD45 RIT. The time point of administration of the second step radiolabeled ligand (radio-DOTA-biotin) significantly impacted the biodistribution of radioactivity in target tissues. Rapid clearance of the FP from the circulation rendered unnecessary the addition of a synthetic clearing agent in this model. These results support proceeding to anti-CD45 PRIT clinical trials for patients with both leukemia and lymphoma.

15.9.2 Radiolabeled Somatostatin Receptor Antagonists

Targeting neuroendocrine tumors expressing somatostatin receptor subtypes (SSTs) with radiolabeled somatostatin agonists is an established diagnostic and therapeutic approach in oncology. While agonists readily internalize into tumor cells, permitting accumulation of radioactivity, radiolabeled antagonists do not, and they have not been considered for tumor targeting. The macrocyclic chelator, DOTA coupled to potent somatostatin receptor-selective peptide antagonists, showed high SST-binding affinity (Ginj et al. 2006). These antagonists labeled many more sites than agonists. Somatostatin antagonist radiotracers, therefore, are preferable to agonists for the in vivo targeting of SST-expressing tumors.

15.9.3 Theophylline Enhances Radioiodide Uptake by Cancer

Radioactive iodine is used for the diagnostic imaging of differentiated thyroid cancer as well as for the ablation or residual thyroid tissue and microscopic metastases. Iodide is incorporated into the cells through endogenous natrium (sodium)/iodide symporters (NIS) that are located at the plasma membrane of the thyroid follicular cells and co-transport Na^+ and I^- ions. The transmembrane sodium gradient maintained by Na^{+}/I^- ATPase is the driving force of iodide uptake enabling intracellular iodide concentrations to reach 20–40 times greater than the background tissues.

Theophylline has been shown to augment radioiodide uptake in breast cancer cells and NIS gene-transduced cancer cells through the upregulation of NIS

expression (Yoon et al. 2009). Therefore, further investigations are warranted to explore the potential utility of this phenomenon for enhancing radioiodide-based imaging and therapies of NIS gene-transduced cancer cells.

15.10 Strategies for Drug Delivery in Cancer

Various strategies for drug delivery in cancer are listed in Table 15.4 and are described in the following text.

15.10.1 Direct Introduction of Anticancer Drugs into the Tumor

15.10.1.1 Injection into the Tumor

Direct injection of an anticancer drug into the tumor aims to achieve a higher local concentration to be reached without systemic toxicity. An example is injection of antineoplastic therapies into malignant brain tumors. Limitations of this approach are the inaccessibility of some tumors and multiple metastases. To reach cancer cells in optimal quantities, therapeutic agents must be delivered to tumors through their imperfect blood vascular system, cross vessel walls into the interstitium, and penetrate multiple layers of tissue. Strategies to enhance drug penetration have potential to improve therapeutic outcome. The development of multicellular layers (MCLs), in which tumor cells are grown on a semipermeable Teflon support membrane, has facilitated quantification of drug penetration through solid tissue. Penetration of commonly used anticancer agents (paclitaxel, doxorubicin, methotrexate, and 5-FUl) through MCLs derived from tumor cell lines is significantly greater through the round (loosely packed) than through the epithelioid (tightly packed) sublines (Grantab et al. 2006). In MCLs treated with doxorubicin, greater survival is observed in the tightly packed cell lines than in the loosely packed cell lines. Impaired penetration of anticancer agents through MCLs derived from the tightly packed cell lines and relative resistance to killing of cells within them by doxorubicin treatment strengthen the role of tumor cell adhesion and packing density as contributing to drug resistance.

Treatment of head and neck cancer involves delivery of the therapeutic agents by direct injection into the tumor lesions. This locoregional therapy allows adaptation of conventional chemotherapy agents, such as bleomycin and cisplatin, to achieve higher drug concentrations in the tumor while avoiding severe systemic toxicities. Novel therapies can be administered through tumor injection, such as gene transfer, photosensitization, and biologic response modifiers.

An important development in intratumor administration of anticancer therapy is the delivery of Ultra Interferons developed by PBL InterferonSource through a

Table 15.4 Strategies for drug delivery in cancer

Strategies
Direct introduction of anticancer drugs into tumor
Injection directly into the tumor
Tumor necrosis therapy
Injection into the arterial blood supply of cancer
Local injection into the tumor for radiopotentiation
Localized delivery of anticancer drugs by electroporation (electrochemotherapy)
Local delivery by anticancer drug implants
Systemic delivery targeted to tumor
Heat-activated targeted drug delivery
Innovations in systemic delivery by vascular route
Tissue-selective drug delivery for cancer using carrier-mediated transport systems
Tumor-activated prodrug therapy for targeted delivery of chemotherapy
Pressure-induced filtration of drug across vessels to tumor
Promoting selective permeation of the anticancer agent into the tumor
Two-step targeting using a bispecific antibody
Site-specific delivery and light activation of anticancer proteins
Drug delivery targeted to blood vessels of tumor
Antiangiogenesis therapy
Angiolytic therapy
Drugs to induce clotting in blood vessels of tumor
Vascular-targeting agents
Special formulations and carriers of anticancer drugs
Albumin-based drug carriers
Pegylated liposomes (enclosed in a PEG bilayer)
Fatty acids as targeting vectors linked to active drugs
Carbohydrate-enhanced chemotherapy
Microspheres as drug delivery system in cancer therapy
MAbs
Nanoparticle-mediated drug delivery
PEG technology
Single-chain antigen-binding technology
Delivery of proteins and peptides for cancer therapy
Transmembrane drug delivery to intracellular targets
Transduction of proteins and peptides
Cytoporter
Receptor-mediated endocytosis
Vitamins as carriers for anticancer agents
Biological therapies
Genetically modified bacteria
Oncolytic viruses
Cell therapy
Gene therapy
Antisense therapy
RNA interference

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proprietary Sustained-Release Protein Delivery (SuRe-PD™) technology. It delivers interferon directly to tumors and releases it slowly over time. Interferon released into a tumor stimulates the immune system to seek out and destroy tumors and stray cancerous cells throughout the body. Another advantage is that Ultra Interferon may

require injection into the tumor only once or twice a month whereas usual injections of interferons are given three times a week for several months.

15.10.1.2 Antineoplastic Drug Implants into Tumors

Usually constructed with insoluble polymers, antineoplastic drug implants provide a constant local supply of the chemotherapeutic agent without systemic toxicity. The disadvantage is that the implants require surgical procedure for repeat insertion or for removal once they are no longer needed. This disadvantage is removed in Fibrasorb, which consists of material that is biodegradable. These local implants should be distinguished from implants of drugs for long-term systemic effect.

15.10.1.3 Tumor Necrosis Therapy

An example of tumor necrosis therapy (TNT) is Peregrine Pharmaceuticals' Cotara (131I-chTNT-1/B), a chimeric modified TNT conjugated to a radioisotope ^{131}I. It is designed to deliver high doses of radiation directly to the core of the tumor by convection-enhanced delivery while causing minimal damage to surrounding tissue. TNT-based drugs are capable of carrying a wide variety of therapeutic agents to the interior of solid tumors. It has been tested in phase II clinical trials in prostate cancer and is in phase II clinical trials for glioblastoma multiforme.

Instead of targeting living cancer cells, TNT targets dead and dying cells. These cells account for up to 50 % of the mass of a tumor and are found primarily at the tumor core. The drug binds to DNA or DNA-associated proteins, such as histones, found within the nucleus of every cell. The drug is not able to discriminate between DNA found in living cells and DNA found in dead cells but is only able to bind to DNA in cells having porous nuclear and cellular membranes. Because porosity is a property uniquely associated with dead and dying cells, the DNA functions as a highly abundant but selective target. Once concentrated in necrotic regions throughout the tumor, the drug is able to bombard neighboring living tumor cells with beta radiation.

Each successive treatment with TNT kills more tumor cells, thereby increasing the necrotic area of the tumor. Thus, TNT becomes more effective upon subsequent doses, contrary to conventional chemotherapy, which may become less effective with subsequent doses as a result of increased drug resistance. Additionally, because radioactive isotopes have a large killing radius of 100–300 cell layers around the isotope, TNT can be effective in any areas of the tumor having small pockets of necrosis surrounded by viable tumor cells.

Oncolym® (Peregrine Pharmaceuticals) is the trade name for the radioimmuno-conjugate formed when the Lym-1 MAb is attached to ^{131}I, which has a number of advantages as a therapeutic radionuclide. The primary potential advantage is that beta radiation emissions from the isotope (the energy that kills the cancer) penetrate several millimeters through tissue killing some 300 cells layers around the anti-body–isotope conjugate. This makes the radioimmunoconjugate therapy potentially

effective against tumors, because it negates the need to target each and every cancer cell individually. Other potential advantages are that iodine is cheap to produce, abundant, requires simple radiolabeling procedures, has a long half-life, has well-characterized dosimetry, and can be imaged.

15.10.1.4 Injection into the Arterial Blood Supply of Cancer

Regional perfusion of the limb or organ containing cancer by way of chemotherapy through intra-arterial injection is a well-known technique in practice. For some organs such as the brain and the lungs, it is still experimental. Innovations are still being made in regional perfusion.

Tumor necrosis factor-alpha (TNF-α) is an immunomodulatory cytokine that has exhibited antitumor activity in a variety of experimental systems. However, the toxicities associated with systemic administration of TNF-α have limited its clinical utility and have led to the investigation of targeted delivery techniques with the ability to present the TNF-α dose directly to the vascular bed of the tumor. The intra-arterial (IA) administration of TNF-α to patients with liver metastases represents one such approach. Studies in rat models have shown improved antitumor activity with the intra-arterial administration of TNF-α over the intravenous route in a regionally confined mammary adenocarcinoma (Badgwell et al. 2003). Intra-arterial administration of biologic response modifiers like TNF-α may therefore be a useful approach for the hepatic chemoembolization of breast adenocarcinomas metastatic to the liver.

Delcath Systems Inc. has developed a system to deliver high-dose chemotherapy directly to an organ or body region. The procedure is repeatable and less invasive than traditional ways of performing isolated perfusion to specific body organs or regions. The system isolates the liver from the general circulation to administer chemotherapy and other therapeutic agents directly to the liver. The blood can be cleansed of the chemotherapy before its return from the liver into the patient's circulatory system. This protects other parts of the body from the harmful side effects of chemotherapy and will allow higher dosages of chemotherapy to be administered to the liver than can be administered with conventional intravenous delivery. In a National Cancer Institute study, melphalan was delivered to the liver via the hepatic artery in patients with unresectable hepatic malignancies. Sixty percent of the evaluable cancer patients treated with high-dose therapy through the Delcath system experienced antitumor activity, with over half of the responding patients achieving tumor shrinkage greater than 50 %.

Transarterial embolization of branches of the hepatic artery with biocompatible 90Y-labeled microspheres (SIR-Spheres) is a local treatment modality for patients with liver tumors. Initial clinical experience confirms that selective internal radiation therapy using SIR-Spheres is feasible as a promising local therapeutic approach in patients with nonresectable liver tumors and has an acceptable toxicity profile (Pöpperl et al. 2005).

15.10.1.5 Electrochemotherapy

Electrochemotherapy is a localized delivery of anticancer drugs by electroporation. The main aim of this approach is to deliver chemotherapeutic substances directly to the tumor and spare the patient from the adverse effects of systemically administered chemotherapy. Electroporation for this application has been developed by Genetronics with a grant from the US National Cancer Institute (NCI). Treatment involves injecting chemotherapeutic drugs into the tumor, allowing them to diffuse throughout, and then applying pulsed electrical fields. It is most suitable for tumors of the skin, such as basal-cell carcinoma. It has been used to deliver the anticancer drug bleomycin tumors into tumors leading to complete remission. The results are comparable to that of surgery, but electroporation is a tissue-sparing procedure (only the tumor undergoes necrosis) that does not lead to as much scarring as surgical procedures do. The tumor antigens from the dying cells may stimulate systemic immune response with shrinking of untreated tumors at other sites. Further clinical trials are planned, and the use of cytokines such as IL-2 in combination with electrochemotherapy is under consideration. This approach is currently limited to local disease and does not cure patients with dispersed metastatic tumors.

Electrochemotherapy is effective in a variety of skin cancers. This treatment represents an excellent alternative to standard surgery or radiotherapy, with an outpatient-based treatment applied in one to three sessions. The major impact was obtained in basal-cell carcinoma, but electrochemotherapy is a useful palliative therapy in melanoma, breast cancer, or SCC. More experience and longer follow-up are required to determine long-term results.

15.10.1.6 Pressure-Induced Filtration of Drugs Across Vessels to the Tumor

A solid tumor is composed of a population of cells that is expanding as a result of cell division. With dense cell packing, the solid matrix of the interstitial tissue is subject to residual stress. The compromised circulation and elevated IFP within tumors are major barriers to adequate and uniform penetration of macromolecules such as MAbs. Increase of tumor microvascular pressure (MVP) and associated changes in IFP could enhance macromolecular delivery into a solid tumor. Alteration of tumor MVP by either periodic injection or continuous infusion of angiotensin II (AII) has been shown to enhance transvascular fluid filtration, leading to a 40 % increase in total uptake of the specific antibody within 4 hours of its administration.

The periodic injection of AII may not be directly applicable because of potential side effects. A more clinically feasible approach to increasing transmural pressure is to reduce tumor IFP, a feat that can be achieved by modulation of the tumor extracellular matrix. For instance, researchers have found that by blocking the integrin links between interstitial matrix and cells, it is possible to reduce IFP and to increase tissue fluid content. Irradiation also can reduce IFP in tumors.

15.10.2 In Situ Production of Anticancer Agents in Tumors

The diverse health benefit effects of garlic include its anticancer activity. The active molecule in garlic, called allicin, is able to destroy cancerous cells by inhibiting telomerase activity and induction of apoptosis. Scientists at the Weizmann Institute in Israel have recently overcome one of allicin's major drawbacks—the fact that it is toxic to normal, healthy cells. They designed a delivery system that enables the destruction of malignant cells without causing damage to the adjacent healthy ones. The new system successfully produces allicin specifically at the site of lymphoma and leukemic cancerous cells. Preliminary results are very encouraging; they show that malignant cells are destroyed in mice that have a type of lymphoma while healthy cells remain intact.

15.10.3 Selective Destruction of Cancer Cells

A problem with conventional chemotherapy or radiotherapy is that damage is not limited to cancer cells but involves normal cells as well. It is easy to kill cells in vitro, and many new anticancer drugs are being discovered. However, it is difficult to selectively kill cancer cells in vivo without harming normal cells. Even though some success is achieved in animal experiments, it is difficult to translate these findings into practical management of cancer patients. Strategies for selective destruction of cancer in vivo are as follows:

- Drugs for selective disruption of cancer metabolism: sphingolipids
- Hyperbaric oxygen (HBO) as adjunct to radiotherapy
- Genetically engineered bacteria for selective destruction of cancer
- Use of MAbs to selectively target anticancer agents to receptors on cancer cells
- Targeting response to transformation-induced oxidative stress
- Targeting enzymes to prevent proliferation of cancer cells

15.10.3.1 Sphingolipids

Cancer cells are sensitive to nutrient limitation because cancer cell's ability to generate ATP is compromised under these conditions. In addition, most cancer cells have defects in autophagy, the catabolic process that provides nutrients from internal sources when external nutrients are unavailable. In contrast, normal cells can adapt to the nutrient stress that kills cancer cells by becoming quiescent and catabolic. A study has shown that FTY720, a water-soluble sphingolipid drug that is effective in many animal models of cancer, selectively starves cancer cells to death by downregulating nutrient transporter proteins (Romero Rosales et al. 2011). Consistent with a bioenergetic mechanism of action, FTY720 induced autophagy of

cancer cells but normal cells were protected. AAL-149, an FTY720 analog that lacks FTY720's dose-limiting toxicity, also triggered transporter loss and killed patient-derived leukemia cells while sparing cells isolated from normal donors. Because FTY720 analogs target the metabolic profile of cancer cells rather than specific oncogenic mutations, they should be effective against several tumor types, particularly in combination with drugs that inhibit autophagy.

15.10.3.2 Hyperbaric Oxygen

HBO, i.e., oxygen under higher than atmospheric pressure, is used for the treatment of several disorders. HBO has been investigated as an adjunct to radiotherapy of cancer. It is well recognized that hypoxia influences the response of cells and tissues to radiation and increases the resistance of cancer to radiotherapy requiring higher radiation doses that can normal tissues. HBO is considered to be the most effective method for counteracting tumor hypoxia for enhancing the effect of radiotherapy on cancer, but this approach has been shown to be effective in only some types of cancer, e.g., glioblastoma multiforme (Jain 2009). In spite of several studies, the controversy has not been resolved. Combination of antineoplastic agents and HBO induces dual injury to the mitochondrial respiration and cell membranes. HBO can be added to regimes combining radiotherapy with chemotherapy. Concomitant HBO enhances the effects of 5-fluorouracil on malignant tumors, but no clinical trials have been done to evaluate this combination.

15.10.3.3 Targeting Response to Transformation-Induced Oxidative Stress

Malignant transformation is often associated with enhanced cellular stress and DNA damage. Cancer cells adapt to this stress to survive and may become dependent upon non-oncogenes that do not ordinarily perform such a vital function in normal cells. Therefore, targeting this non-oncogene dependency may result in selective death of cancer cells. A cell-based small-molecule screening and quantitative proteomics approach led to the unbiased identification of piperlongumine, a small molecule that selectively kills cancer cells but not normal cells (Raj et al. 2011). Piperlongumine increases the level of reactive oxygen species (ROS) and apoptotic cell death in both cancer cells and normal cells engineered to have a cancer genotype, irrespective of p53 status, but it has little effect on normal cells. Significant antitumor effects were observed in mouse xenograft tumor models treated with piperlongumine, but no toxic effects were observed in normal mice. Moreover, piperlongumine inhibits the growth of spontaneous breast cancers in mice. These findings show that ability a small molecule can selectively induce apoptosis in cells that have a cancer genotype by targeting a non-oncogene dependency acquired through the expression of the cancer genotype in response to oxidative stress induced by malignant transformation.

15.10.3.4 Targeting Enzymes to Prevent Proliferation of Cancer Cells

CFI-400945 has been designed by a team of scientists in Canada and China to specifically prevent proliferation of cancer cells but not damage normal cells. It targets an enzyme called PLK4, which plays a critical role in cell division, especially in cancer cells. Cells in genomically unstable cancers can have scores more chromosomes than the 46 present in normal cells, and these malignant cells rely on PLK4 to be able to continue to proliferate out of control. Targeting this enzyme would prevent survival of these cells. Animal experimental studies have been completed, and FDA permission to start human clinical trials is pending with expected go ahead in the fall of 2013. Initial trial with the drug will be a study in patients with breast or ovarian cancers to determine a safe dose.

15.11 Targeted Drug Delivery in Cancer

Delivery of anticancer therapeutic agents to solid tumors is problematic. The concept of targeted delivery is important for the development of new anticancer agents. Targeting implies that the purpose of achieving selectivity and specificity is being applied in the design of the therapeutic approach. Targeting can be external or internal. External targeting of a tumor may be carried out by the use of a nonspecific agent such as radiation, which is directed at the tumor to achieve a selective antitumor effect. Internally targeted therapeutic agents act in three different ways:

1. Selective uptake by the tumor-targeted delivery.
2. Selective expression/activation in tumor microenvironments (e.g., the use of protease-sensitive toxins whose cytotoxic action is manifested after cleavage by tumor-associated proteases).
3. Selective action against molecular targets expressed in tumors. Conventional small-molecular drugs are usually targeted through selective action on the molecular machinery of the targeted cells.

Targeting approaches can be passive tumor targeting and active tumor targeting:

1. Passive tumor targeting involves transporting anticancer agents through the bloodstream to tumor cells using a "carrier" macromolecule. Different carrier macromolecules are being investigated as each provides advantages such as specificity and protection of the anticancer drug from degradation due to their structure, size, and particular interactions with tumor cells.
2. Active tumor targeting involves attaching a receptor molecule to the anticancer drug to create a new "targeted" agent that will actively seek a complementary surface molecule to which it binds. Targeting the anticancer agent to the affected cells should allow more of the anticancer drug to enter the tumor cell thus amplifying the response to the treatment and reducing the toxic effect on normal tissue. Examples of active targeting fragments include antibodies, growth factors, and vitamins.

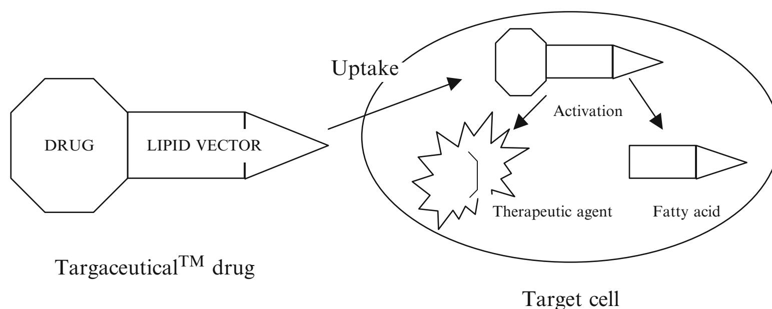

Fig. 15.1 Mechanism of action of Targaceutical drugs

15.11.1 Affibody Molecules for Targeted Anticancer Therapy

Affibody® molecules (Affibody AB) are a novel class of small (approximately 7 kDa) phage display-selected affinity proteins, based on the B-domain scaffold of staphylococcal protein A. A radiolabeled anti-HER2 Affibody molecule was fused with an albumin-binding domain (ABD) and labeled with the low-energy beta-emitter ^{177}Lu. The radiolabeled conjugate displayed specific binding to HER2-expressing cells and good cellular retention in vitro. In vivo, fusion with ABD enabled a 25-fold reduction of renal uptake in comparison with the nonfused dimer molecule. The in vivo association to serum albumin increases the half-life of the Affibody® molecule, thereby optimizing the amount of drug loaded onto the tumor. Furthermore, the biodistribution showed high and specific uptake of the conjugate in HER2-expressing tumors. Treatment of SKOV-3 microxenografts with this conjugate completely prevented formation of tumors, in contrast to mice given a radiolabeled non-HER2-binding Affibody molecule. Thus, fusion with ABD improved the in vivo biodistribution, and the results highlight radiolabeled Affibody fused with ABD as a candidate for treatment of disseminated tumors with a high level of HER2 expression (Tolmachev et al. 2007).

15.11.2 Fatty Acids as Targeting Vectors

Targaceutical® technology (Luitpold Pharmaceuticals) uses fatty acids as targeting vectors, chemically linking them to active drugs. The release of the drug is delayed until it is taken up by the target tissue, thereby reducing side effects. The basis of this is that tumors accumulate certain fatty acids in greater amounts than do normal cells and are metabolized over a longer period, providing slow release of the drug. The conjugates are inert but are activated when taken up and metabolized by the tumor cells (see Fig. Fig. 15.1). The linkage between the drug and the fatty acid is cleaved and the activity of the drug is revealed within the cells.

Several compounds have been synthesized by Protarga, but the leading compound is Taxoprexin® injection and is currently in phase III clinical trials. It consists of the taxane class of drugs, which links the fatty acid docosahexaenoic acid (DHA) to the cancer drug. DHA is an omega-3 fatty acid that is essential for proper human development. It is used as a membrane precursor and energy source. DHA is synthesized by many cell types in man but not in sufficient quantities during nutritional stress. The additional required DHA is provided by the diet. The safety of DHA has been validated in extensive human nutrition trials. DHA is approved in Europe for use in infant formula, and it is marketed in the USA as a nutritional supplement.

15.11.3 Genetic Targeting of the Kinase Activity in Cancer Cells

Assessing the oncogenic potential of individual kinase activities and ensuring that a drug of interest acts by direct inhibition of its putative target kinase is an important goal in anticancer drug discovery. A genetic strategy has been developed to selectively inactivate the catalytic activity of kinases (Arena et al. 2007). This approach generates isogenic cells in which a given kinase gene is expressed but is devoid of enzymatic activity. The MET receptor, which is involved in multiple cancers and is the focus of several therapeutic efforts, was chosen as a model to test this approach. The exon encoding the ATP-binding site of MET was deleted from the genome of colorectal, bladder, and endometrial cancer cells. The derivative isogenic cells expressed a kinase-inactive Met (MET-KD) and were completely unresponsive to its ligand hepatocyte growth factor (HGF), indicating the exclusivity of this ligand–receptor axis. The in vivo tumorigenic potential of MET-KD cells was reduced but could be partially restored by HGF, suggesting that concomitant targeting of the receptor and its ligand should be therapeutically exploited. A selective Met-kinase inhibitor (SU-11274) markedly affected the growth of MET-KD cancer cells. This genetic strategy is not limited to kinase genes but could be broadly applicable to any drug/protein combination in which the target enzymatic domain is known.

15.11.4 Novel Transporters to Target Photosensitizers to Cancer Cell Nuclei

A novel approach has been described that uses modular recombinant transporters to target photosensitizers to the nucleus of cancer cells overexpressing ErbB1 receptors, where their action is most pronounced (Gilyazova et al. 2006). The new generation of transporters consists of (a) epidermal growth factor as the internalizable ligand module to ErbB1 receptors, (b) the optimized nuclear localization sequence of SV40 large T antigen, (c) a translocation domain of diphtheria toxin as an

endosomolytic module, and (d) the *Escherichia coli* hemoglobin-like protein HMP as a carrier module. The modules retained their functions within the transporter chimera: they showed high-affinity interactions with ErbB1 receptors and α/ß-importin dimers and formed holes in lipid bilayers at endosomal pH. A photosensitizer conjugated with the transporter produced singlet oxygen and·OH radicals similar to the free photosensitizer. Photosensitizers–transporter conjugates have >3,000 times greater efficacy than free photosensitizers for target cells and were not photocytotoxic at these concentrations for cells expressing a few ErbB1 receptors per cell, in contrast to free photosensitizers. The different modules of the transporters, which are highly expressed and easily purified to retain full activity of each of the modules, are interchangeable, meaning that they can be tailored for particular applications.

15.11.5 Photodynamic Therapy of Cancer

Photodynamic therapy (PDT) is a cancer treatment which generates tumor kill through the production of singlet oxygen in cells containing a photosensitizing drug when exposed to laser light of a specific wavelength. PDT is used to treat a range of diseases characterized by rapidly growing tissue, including the formation of abnormal blood vessels, such as cancer. The more traditional name for this therapy is photoradiation therapy. Treatment with PDT consists of a two-step process that starts with administration of the drug, or photosensitizer, by intravenous injection. Once the drug enters the bloodstream, it attaches itself to low-density lipoproteins already circulating. As cells undergoing rapid growth require an above-average supply of lipoproteins, the drug reaches these types of cells more quickly and in higher concentrations.

Once the necessary level of concentration is attained, the second step is to activate the drug with a specific dose of light of a particular wavelength. This causes the conversion of normal oxygen found in tissue to a highly energized form called singlet oxygen, which in turn disrupts normal cellular functions. Neither the drug nor the light exerts any effect until combined.

What makes PDT effective and safe is its selectivity. Because the light shines directly at the cells in the targeted tissue where the drug accumulates preferentially, damage to the surrounding tissue is limited. The entire procedure can be performed in a physician's office or on an outpatient basis. The type of light source used in PDT varies according to the condition treated. For cancer, fiber optics is used to deliver light to the internal organs like the lung and esophagus while light-emitting diodes (LED) are used for skin cancer.

Litx™ (Light Sciences Oncology, Inc.) is a combination drug/device product that includes a proprietary flexible LED array and Aptocine™ (talaporfin sodium), a light-activated, water-soluble drug. Aptocine™ has been approved in Japan where it is currently marketed for the treatment of early-stage bronchopulmonary cancer. In a Aptocine™ treatment, the physician inserts the LED array into the tumor

through the skin in a biopsy-like procedure. The physician then injects the patient with Aptocine™, an inert molecule that has no biological activity. When the light source is activated, the light from the array energizes the Aptocine™ molecule, causing conversion of molecular oxygen to singlet oxygen, which kills tissue within a zone surrounding the LED array and shuts down tumor blood supply within that zone. The light source is typically left on for approximately two and one-half hours to maximize the effect on the tumor and to assure continued blood vessel closure. The combination of light-activated drug and light-delivery technology is designed to provide physicians with a simple system containing all treatment-required components included in one single-use package.

15.11.6 Radionuclides Delivered with Receptor-Targeting Technology

Various isotopes of radium are used for cancer therapy. Alpharadin (Algeta ASA), radium-223, is in phase II trials as a potential treatment of skeletal metastases in patients with hormone refractory prostate cancer. It has intrinsic disease-relevant targeting properties, i.e., a natural affinity for bone, as well as moderate photon emission, enabling concurrent imaging. Another isotope, thorium-227, exhibits significant cytotoxicity when targeted to tumor cells. Thorium-227 has a half-life of 18.7 days and like radium-223 is produced from a generator system. The relative long half-life of thorium-227 may enable the administration and targeting of a thorium-227-labeled radioimmunoconjugate. Thorium-227 forms the basis of Algeta's TH-1 receptor-targeting technology, which is designed to enhance the potency of therapeutic antibodies and other tumor-targeting molecules when linked to them. This technology is in preclinical studies for various cancers.

15.11.7 Targeting Ligands Specific for Cancer Cells

A DDS to minimize the uptake of the drug by normal cells and enhance the influx and retention of the drug in cancer cells should include three main components: (1) an apoptosis-inducing agent (anticancer drug), (2) a targeting moiety-penetration enhancer, and (3) a carrier. Such a system has been described, which utilizes camptothecin as an apoptosis-inducing agent and PEG as a carrier (Dharap et al. 2005). Luteinizing hormone-releasing hormone (LHRH) was used as a targeting moiety (ligand) to LHRH receptors that are overexpressed in the plasma membrane of several types of cancer cells and are not expressed detectably in normal visceral organs. The results showed that the use of LHRH peptide as a targeting moiety in the anticancer DDS substantially enhanced the efficacy of chemotherapy, led to amplified apoptosis induction in the tumor, and minimized the side effects of the anticancer drug on healthy organs. The LHRH receptor-targeting DDS did not show in vivo

pituitary toxicity and did not significantly influence the time course or the plasma concentration of luteinizing hormone and its physiological effects on the reproductive functions of mice.

15.11.8 Targeting Abnormal DNA in Cancer Cells

Dicycloplatin (DCP) is a new anticancer platinum agent that has been developed by Bioplatin AG on the basis of molecular biology theories about the characteristics of the abnormal structure of DNA in cancer cells. It is in the shape of "cage" with "molecule switches" specially designed to target and bind with the base of abnormal DNA. DCP's therapeutic effect is due to its ability to target and kill cancerous cells but spare normal cells. Several studies have demonstrated DCP's advantages such as its chemical stability, its efficacy as an anticancer agent, its high safety and low toxicity, and its water solubility. A clinical trial has indicated that DCP's toxic effects on the digestive tract and the kidney are less than those of *cis*-diammine, and its hematological toxicity is less than that of carboplatin. It has also been found that DCP's protein binding rate is low, and over 90 % of the drug is in free form when administered to human beings, and it has a long elimination half-life. It has the potential to replace similar anticancer drugs. It has shown exceptionally good results for liver cancer, bronchial carcinoma, and stomach cancer during the clinical studies.

15.11.9 Targeted Delivery by Tumor-Activated Prodrug Therapy

The basic principle of the targeted delivery approach is that conjugation of the drug to a tumor-specific molecule renders the drug inactive until it reaches a target site. Once at the tumor site, the conjugated drug binds to the surface tumor cells and is processed further to restore its original potency. Thus the drug conjugates can be considered as tumor-activated prodrugs (TAPs). Whereas most conventional prodrugs are converted to active drugs by mechanisms such as chemical or enzymatic hydrolysis, the activation of TAPs depends on interaction with antigens or receptors found specifically on the surface of tumor cells. The goal of TAP cancer treatment has not been realized as yet, but considerable experience gained from past failures is helping design a new generation of TAPs. Researchers have remedied the problem of low potency by incorporating drugs that are up to 1,000 times more potent than those used in earlier conjugates. The use of stable disulfide bonds for linking antibody and drug enables the exploitation of antigen specificity of the antibody. These conjugates can be characterized as TAPs that are nontoxic in circulation but are preferentially converted into active drugs upon binding to the tumor and subsequent internalization into the tumor cells. ImmunoGen Inc.'s TAP technology expands the potential of MAbs for cancer therapy by using them to deliver potent cell-killing agents. The first product to be delivered with TAP technology, ADC Kadcyla™, is now approved in the USA (see Chap. 8).

15.11.10 Targeting Glutathione S-Transferase

Telik is developing TELCYTA™ (TLK286), a small-molecule compound, which has a novel “smart” mechanism of activation within cancer cells. It is activated by glutathione S-transferase P1-1 (GST P1-1), an enzyme that is overexpressed in many human cancers. Elevated GST P1-1 levels also correlate with resistance to commonly used chemotherapeutic drugs. Upon activation, TELCYTA™ initiates apoptosis. The compound has been well tolerated in clinical trials, perhaps due to its novel mechanism of action, and shown evidence of tumor responses and prolongation of survival. A randomized phase 3 clinical trial is underway with in ovarian cancer patients whose disease has progressed following platinum-based chemotherapy and one second-line treatment. The multinational, designated the ASSIST-1 (ASsessment of Survival in Solid Tumors-1) trial, is designed to evaluate whether TELCYTA™ treatment reduces the risk of death, leading to an increase in survival, as compared to the control group treatments. Phase II/III clinical trials are in progress with TELCYTA™ in non-small-cell lung, ovarian, and breast cancer and in combinations with standard chemotherapeutic drugs.

15.11.10.1 Targeted Drug Delivery of Anticancer Agents with Controlled Activation

The future of drug delivery for cancer lies in finding ways to target a drug specifically to a diseased cell, or even a molecule within that cell, while leaving healthy cells and molecules unharmed. Some of current strategies for targeted drug delivery aim at getting the drug into the organ affected by cancer. They do not necessarily tackle the challenge of getting the drug into the diseased cells and sparing healthy cells. One approach involves administration of an inactive drug to the patient, which zeros in on the target cells followed by injection of a separate compound that induces the drug to activate only when it reaches its target. This method can also be used to create more precise images of diseased tissue.

15.11.11 Targeted Delivery of Anticancer Agents with ReCODE™ Technology

Ambrx Inc.’s ReCODE™ technology (reconstituting chemically orthogonal-directed engineering) provides control over the site-directed placement of chemistry that is designed and demonstrated to be non-disruptive to protein function. The technology is described on the web site of the company (http://www.ambrx.com/). Through directed evolution and selection, specialized orthogonal tRNA synthetases are evolved to selectively and specifically aminoacylate, a similarly orthogonal amber codon-suppressing tRNA with unique, chemically specified amino acids. The cell’s

translational apparatus then incorporates the Ambrx amino acid into the elongating peptide sequence at positions designated by the amber codon. ReCODE™ technology has broad application across multiple protein classes, including cytokines, peptides, and antibodies, and across multiple therapeutic areas, including cancer.

15.11.12 Transferrin-Oligomers as Targeting Carriers in Anticancer Drug Delivery

Cross-linking of transferrin receptor (TfR) induced by oligomeric Tf binding alters the intracellular trafficking of Tf–TfR complexes, redirects them out of the recycling pathway, and targets them to intracellular degradation. The alteration of TfR-trafficking facilitates the intracellular release of the drug from the Tf-conjugated form. Consequently, Agg-Tf-MTX (methotrexate) is more effective than Mono-Tf-MTX as a TfR-mediated antiproliferative agent in tumor cells, as well as in MTX-resistant transport-deficient cells. Therefore, Tf-oligomers are potentially effective TfR-targeting carriers for intracellular delivery of anticancer drugs.

15.11.13 Tumor Targeting with Peptides

Tumor targeting with peptides is based on the finding that receptors for many regulatory peptides are overexpressed in tumor cells, compared to normal tissues. Tumor targeting with hormone peptides provides a basis for the development of new diagnostic and therapeutic approaches for cancer (Mező and Manea 2010).

Approximately 80 % of human ovarian and endometrial cancers and 50 % of breast cancers express GnRH and its receptor as part of an autocrine regulatory system. Cytotoxic GnRH analogs have been developed, where doxorubicin (DOX) was covalently coupled to GnRH analogs. These compounds have superior antitumor effects in cancers expressing GnRH receptors as compared with native doxorubicin and enable targeted cytotoxic chemotherapy of gynecologic and breast cancers. Novel therapeutic modalities for breast, prostate, and ovarian cancer consist of the use of targeted cytotoxic analogs of LHRH containing DOX or 2-pyrrolino-DOX. The same radicals have been incorporated into cytotoxic analogs of somatostatin which can be also targeted to receptors for this peptide in prostatic, mammary, ovarian, and renal and lung cancers, brain tumors, and their metastases. A targeted cytotoxic analog of bombesin containing 2-pyrrolino-DOX has also been synthesized and successfully tried in experimental models of prostate cancer, small-cell lung carcinoma, and brain tumors. The development of these new classes of peptide analogs should lead to a more effective treatment for various cancers. Experimental human gastric carcinomas that express high-affinity subtype 1 bombesin receptors can be suppressed by cytotoxic somatostatin analog AN-238. These findings suggest that this class of targeted compounds should be considered for the therapy of patients with advanced gastric carcinoma.

15.11.14 Ultrasound and Microbubbles for Targeted Anticancer Drug Delivery

In fluids, pressure-driven cavitation bubbles have a nonlinear response that can lead to extremely high core-energy densities during the collapse phase, a process underpinning phenomena such as sonoluminescence and plasma formation. Encapsulated microbubbles are commonly used to improve echo generation in diagnostic ultrasound imaging. It is possible that such cavitation could also lead to jet-induced tissue damage. Certainly ultrasonic irradiation (insonation) of cells in the presence of microbubbles can lead to enhanced membrane permeabilization and molecular uptake (sonoporation). There is direct observational evidence that shows the energetic micrometer-scale interactions between individual cells and violently cavitating shelled microbubbles. Gas bubbles injected intravenously are known to cluster around the cancerous cells. These data suggest that sonoporation at higher intensities can be used to target and destroy cancer cells.

The ultrasound treatment could be used to refine chemotherapy. Gas bubbles react to the ultrasound by instantaneously inflating like a balloon. The shell of the inflated bubble deforms to develop a fast moving spike directed back into the nearby cancerous cell. When the spike hits the cell membrane, it punches through it like a bullet, creating a tiny "entrance wound." The gas bubbles injected into the cancer patient can be coated with anticancer drugs that then enter the punctured cancer cells. The membranes appear to be able to reseal themselves soon afterwards, effectively locking any drug molecules inside. The drugs are therefore targeted to only the cancer cells in a one shot process, rather than repeatedly flooding the patient's entire body. Such coated bubbles have already been developed. This should dramatically reduce the patient's recovery time and the associated pain and suffering of surgery and chemotherapy.

New strategies to detect tumor angiogenesis and monitor response of tumor vasculature to therapy are needed. Peregrine Pharmaceuticals' Vascular Targeting Agent technology using contrast ultrasound imaging with microbubbles targeted to tumor endothelium offers a noninvasive method for monitoring and quantifying vascular effects of antitumor therapy. The microbubbles are tiny lipid or albumin shells filled with an inert gas that have a well-established safety record as contrast agents for ultrasound imaging applications, and they are currently widely used in cardiovascular medicine. Targeted microbubbles conjugated to MAbs were used to image and quantify vascular effects of two different antitumor therapies in pancreatic tumor-bearing mice treated with anti-vascular VEGF MAbs and/or gemcitabine (Korpanty et al. 2007). Video intensity from targeted microbubbles correlated with the level of expression of the target (CD105, VEGFR2, or the VEGF–VEGFR complex) and with microvessel density in tumors under antiangiogenic or cytotoxic therapy. It was concluded that targeted microbubbles represent a novel and attractive tool for noninvasive, vascular-targeted molecular imaging of tumor angiogenesis and for monitoring vascular effects specific to antitumor therapy in vivo. This information could allow oncologists to modify patient treatment regimens soon

after starting therapy, so that nonresponders could be switched to other therapies that might be more effective for them. The clinical development of contrast agents is typically faster than for therapeutics, and clinical trials of this approach could be feasible within 12–18 months. The potential of the approach is enhanced by the fact that the targeted microbubbles are "read" using ultrasound technology, which is widely available in most physicians' offices and is minimally invasive, safe, and cost-effective. The personalized medicine made feasible by this approach has the potential to increase the efficacy of cancer regimens, reduce side effects from ineffective treatments, and improve the overall cost-effectiveness of cancer therapy.

15.11.15 Ultrasound for Targeted Delivery of Chemotherapeutics

Effects of low-frequency ultrasound exposure in combination with liposomally encapsulated doxorubicin (Caelyx) and Plurogel encapsulated fluorouracil (5-FU) have been investigated in nude mice inoculated with a human colon cancer cell line, at various concentrations (Myhr and Moan 2006). Non-hyperthermic ultrasound treatment was shown to significantly increase the effect of liposomally encapsulated cytostatic drugs on tumor growth. Synergetic effects were larger for low drug concentrations, indicating that the approach may benefit patients for whom chemotherapeutic treatment have limited effect or for whom drug concentrations have to be restricted due to toxicity.

Pulsed high-intensity focused ultrasound (HIFU) could effectively serve as a source of hyperthermia with thermosensitive liposomes to enhance delivery and efficacy of doxorubicin in tumors. Combining low-temperature heat-sensitive liposomes with noninvasive and nondestructive pulsed-HIFU exposures has been shown to enhance the delivery of doxorubicin and, consequently, its antitumor effects (Dromi et al. 2007). This combination therapy could potentially produce viable clinical strategies for improved targeting and delivery of drugs for treatment of cancer.

15.11.16 Vitamin B12 and Folate for Targeting Cancer Chemotherapy

It is well known that vitamin B12 and folic acid (a part of B12 family) are essential for tumor growth, and as a result, receptors for these vitamins are upregulated in certain tumors. Vitamin B12 receptor overexpression occurs in breast, lung leukemic cells, lymphoma cells, bone, thyroid, colon, prostate, and brain cancers and some other tumor lines, while folate receptor overexpression occurs in breast, lung, ovarian, endometrial, renal, colon, and brain tumors.

Vitamin B12 oral drug delivery. Access Pharmaceutical's technology involves the conjugation of vitamin B12 to a polymer to which is also attached the drug to be delivered or attached to a nanoparticle in which the drug is incorporated. Extensive preclinical work has been conducted evaluating vitamin B12 oral drug delivery technology, which has demonstrated the potential for this receptor-based oral transport delivery system. Vitamin B12 utilizes the body's natural transport system for vitamin B12. This receptor-mediated process actively transports vitamin B12 from the gut to the bloodstream. Access' scientists have found that the attachment of vitamin B12 to drugs, polymers containing drugs, and even drugs encapsulated in nanoparticles provides formulations, which are absorbed into the body using vitamin B12 uptake mechanism. The Access technology combines the amplification effects of macromolecular transport with active targeting. It is specifically focused on using vitamin B12 and folate to more effectively target anticancer drugs to solid tumors.

Folate-based drug delivery. Receptor for folic acid constitutes a useful target for tumor-specific drug delivery, primarily because (1) over 30 % of cancers overexpress the folate receptor, (2) access to the folate receptor in those normal tissues that express it can be severely limited due to its location, and (3) folate receptor density appears to increase as the stage/grade of the cancer worsens. Thus, cancers that are most difficult to treat by classical methods may be most easily targeted with folate-linked therapeutics. The presence of folate receptors, which are overexpressed in cancer cells but not found in significant quantities in normal cells, guarantees that this new drug delivery system targets only cancer cells.

There are two routes by which folic acid enters cells. The major route is via the reduced folate carrier. However, it does not allow drug–folate conjugates to pass across into the cell. The other route is via the folate receptor. Drug–folate conjugates can enter the cell via this route. Folate-based drug delivery system has the following advantages over drug delivery technologies based on MAbs:

- Folate is much smaller than monoclonals (molecular weight of folate is 441, while many monoclonals' molecular weights are 150,000), allowing improved tumor access.
- Folate is inexpensive compared to MAbs.
- Folate is a natural substance and does not elicit an immune response from the body. It can, therefore, be administered many times. Many MAbs, on the other hand, prompt an immune response that destroys the MAB and eliminates any chance that the MAb can be used again.
- Folate is very stable during synthesis and storage, and conjugation chemistry and product purification are simple.
- The affinity of folate for its receptor on cancer cells is higher (KD is approximately 10–9 M) than MAb–antigen interactions.
- Because the folate receptors on cancer cells recycle, a large number of folate–anticancer agent conjugates can be absorbed by the cell.

Membrane transport is also a critical determinant of the antitumor activity of antifolate therapeutics (methotrexate, Tomudex) used in cancer chemotherapy, and

impaired uptake of antifolates is a frequent mode of drug resistance. Since the folate receptor-mediated endocytosis pathway is a nondestructive route of cellular internalization, it allows for the delivery of hydrolytically sensitive proteins and genes. Like current cancer treatments such as tamoxifen that are based on hormone receptors, folate receptor-mediated cancer treatments could be used to target drugs and diagnostic agents to cancer cells that abundantly express the folate receptor.

To exploit these peculiarities of folate receptor expression, folic acid has been linked to both low molecular weight drugs and macromolecular complexes as a means of targeting the attached molecules to malignant cells. Conjugation of folic acid to macromolecules has been shown to enhance their delivery to folate receptor-expressing cancer cells in vitro in almost all situations tested. Folate-mediated macromolecular targeting in vivo has, however, yielded only mixed results, largely because of problems with macromolecule penetration of solid tumors. Nevertheless, prominent examples do exist where folate targeting has significantly improved the outcome of a macromolecule-based therapy, leading to complete cures of established tumors.

PEG-coated biodegradable nanoparticles can be coupled to folic acid to target the folate-binding protein, which is the soluble form of the folate receptor that is overexpressed on the surface of many tumor cells. The specific interaction between the conjugate folate nanoparticles and the folate-binding protein has been evaluated by surface plasmon resonance and confirmed a specific binding of the folate nanoparticles to the folate-binding protein. Thus, folate-linked nanoparticles represent a potential new drug carrier for tumor cell-selective targeting.

15.11.17 Cell-Based Drug Delivery in Cancer

Cells can be used as vehicles for drug delivery. Various types of cells used for drug delivery include red blood cell (RBC), white blood cell (WBC), and stem cells. Implantation of encapsulated cells that are genetically modified to secrete therapeutic proteins is another method of cell-based drug delivery.

Cell-mediated delivery is a therapeutic as well as a diagnostic strategy for cancer. The cytotoxic activity of the cell carriers can be combined with site-specific delivery of anticancer agents, which can be packed into the carriers to reduce cytotoxicity to normal tissues. Nanoparticles have been combined with cell-based delivery systems. A technique has been described to create nanoparticulate cellular patches that remain attached to the membrane of cells for up to 2 days and retain their inherent tumoritropic properties as shown using a tumor model in a 3D extracellular matrix (Cheng et al. 2010). Various cells that have been evaluated as carriers for delivery of anticancer agents include MSCs, RBCs, mononuclear phagocytes, and bacterially derived minicells.

Mononuclear phagocytes have been used as “Trojan horses” for gold nanoparticle transport into hypoxic centers of tumors, which were destroyed by irradiation with near-infrared light (Choi et al. 2007). Macrophages can also act as a cellular vehicle for 5-FU encapsulated in oligomannose-coated liposomes cased in

magnetic nanoparticles followed by treatment with an alternating magnetic field, which led to the release of 5-FU from the macrophages. Mononuclear phagocytes can also be used for delivery of therapeutic DNA constructs into tumors.

15.11.17.1 Red Blood Cells as Vehicles for Drug Delivery

Drugs can be encapsulated in RBCs, which represent naturally designed carriers for intravascular drug delivery, characterized by unique longevity in the bloodstream, biocompatibility, and safe physiological mechanisms for metabolism (Muzykantov 2010). RBCs have been used as carriers for anticancer agent 5-fluorouracil (5-FU) by intravenous injection to treat malignant ascites, resulting in an increase of survival time in mice (Wang et al. 2010). Several protocols of infusion of RBC-encapsulated drugs are being explored in patients. Delivery of drugs, particularly those targeting phagocytic cells and those that must act within the vascular lumen, may benefit from carriage by RBCs. Two strategies for RBC drug delivery are (1) encapsulation into isolated RBCs ex vivo followed by infusion in compatible recipients and (2) coupling of drugs to the surface of RBCs. RBC drug delivery by injection of therapeutics conjugated with fragments of antibodies provides safe anchoring of cargoes to circulating RBC, without need for ex vivo modification and infusion of RBC.

15.11.17.2 Cells as Vehicles for Gene Delivery

AC133+ progenitor cells (APC) can be used as both gene delivery vehicles and cellular probes for MRI). A study has shown that superparamagnetic iron oxide (SPIO)-labeled APCs can carry the human sodium iodide symporter (hNIS) gene to the sites of implanted breast cancer in mouse model (Rad et al. 2009). In vivo real-time tracking of these cells was performed by MRI and expression of hNIS was determined by Tc-99 m scan. Thus genetically transformed, magnetically labeled APCs can be used both as delivery vehicles and cellular probes for detecting in vivo migration and homing of cells. Furthermore, they can potentially be used as a gene carrier system for the treatment of tumors.

15.11.18 Implants for Systemic Delivery of Anticancer Drugs

Generally implants for systemic delivery of anticancer drugs have a more restricted application than do local implantation of anticancer drugs. One reason is that most cancers require a short-term aggressive treatment. However, other cancers such as prostate require long-term treatment.

The best known system consists of ALZA's VIADUR® product (leuprolide acetate implant) incorporating DUROS implant technology, which is used for once-yearly palliative treatment of prostate cancer. The FDA-approved system is

distributed by Bayer Pharmaceuticals. The DUROS implant is a miniature cylinder made from a titanium alloy, which protects and stabilizes the drug inside, using a proprietary formulation technology. Water enters into one end of the cylinder through a semipermeable membrane; the drug is delivered from a port at the other end of the cylinder at a controlled rate.

With the introduction of anticancer proteins and other bioactive molecules, drug-eluting polymer implants will provide an important parenteral route of administration for cancer chemotherapy. They have the potential for minimally invasive, image-guided placement and highly localized drug release. Current trends in the application of preformed and in situ-forming systems as drug-eluting implants for cancer chemotherapy have been reviewed elsewhere (Exner and Saidel 2008). Whether used alone or in combination with other minimally invasive procedures, drug-eluting polymeric implants will play a significant role in the future of cancer management.

15.11.19 Angiogenesis and Drug Delivery to Tumors

A growing tumor needs an increased blood supply for its proliferating cells. Tumor vessels possess unique physiological features that might be exploited for improved drug delivery. For example, targeting of liposomal anticancer drugs to tumor vasculature is increasingly recognized as an effective strategy to obtain superior therapeutic efficacy with limited host toxicity compared with conventional treatments (Lila et al. 2009).

The implications of tumor-related angiogenesis are somewhat complex. Although these new vessels are required to nourish the tumor itself, they are disorganized and abnormal and can actually block therapeutic agents from reaching malignant cells (Jain 2008). The proliferating cancer cells compress both blood and lymphatic vessels within tumors. The findings suggest new strategies for improving the success of cancer treatment. The antiangiogenesis drug bevacizumab (Avastin) acts by reducing both the number and density of blood vessels within tumors, as well as by reducing fluid pressures. This finding is the first clinical confirmation that normalizing the distorted blood supply within tumors could improve the results of therapy. The matrix actually has two components, one that is nearly liquid and a more viscous component that appears to be the most significant barrier to drug delivery. Targeting the viscous matrix component may also improve treatment results. Therapies designed to destroy abnormal blood vessels act to repair them and make the cancer more accessible to chemotherapy.

15.11.20 Antiangiogenesis Strategies

Several antiangiogenesis strategies are used in the treatment of cancer. Drug delivery technologies for antiangiogenesis are shown in Table 15.5.

Table 15.5 Methods of delivery of antiangiogenesis therapies

Systemic delivery orally or by injection and targeting to tumor vasculature
Selective intra-arterial injection into the arteries supplying the tumor
Injection or deposition into the tumor cavity after resection of the tumor
Injection of encapsulated or coated cells releasing angiostatic substances into the tumor
Nanoparticle-based delivery of cytotoxic agents to tumor vasculature
Tumor-specific drug delivery by genetically engineered organisms
Gene therapy, e.g., viral vector-mediated delivery of angiostatin gene
RNAi and antisense antiangiogenesis approach

Delivery of blood-borne molecules and nanoparticles from the vasculature to cells in the tissue differs dramatically between tumor and normal tissues due to differences in their vascular architectures. Two simple measures of vascular geometry—δmax and λ—readily obtained from vascular images, capture these differences and link vascular structure to delivery in both tissue types (Baish et al. 2011). The longest time needed to bring materials to their destination scales with the square of δmax, the maximum distance in the tissue from the nearest blood vessel, whereas λ, a measure of the shape of the spaces between vessels, determines the rate of delivery for shorter times. These findings are useful for evaluating how new therapeutic agents that inhibit or stimulate angiogenesis alter the functional efficiency of the vasculature and more broadly for analysis of diffusion in irregularly shaped domains.

15.11.20.1 Targeting Tumor Endothelial Cells

One approach is based on the observation that tumor endothelial cells can function as targets for the antiangiogenic therapy of cancer. Angiogenesis facilitates formation of metastasis and sustains new tumors. Early tumors recruit endothelial progenitor cells (EPCs) derived from bone marrow (BM), which differentiate into mature BM-derived endothelial cells and incorporate into the lumens of a subset of sprouting tumor neovessels. Specific ablation of BM-derived EPCs with alpha-particle-emitting anti-VE-cadherin antibody markedly impairs tumor growth associated with reduced vascularization (Nolan et al. 2007). RNAi can be used to deliver genes that encourage apoptosis in these EPCs while maintaining a conventional anticancer therapy. This technique holds significant promise for the prevention of tumor development and spreading.

15.11.21 Vascular-Targeting Agents

Vascular-targeting agents are designed to destroy vascular endothelium or induce coagulation of blood and platelet activation both in tumor vessels where division is

taking place and in tumor vessels where it is not. Blood-transporting vessels are destroyed in addition to capillary sprouts. This feature broadens the effect of vascular-targeting agents on the tumor because most vessels are affected and because blood flow is halted in tumor vessels upstream of the thrombosed vessels, even those that lack the target marker. The action of the vascular-targeting agent may therefore be particularly suited to the treatment of larger tumors. Avastin (Roche/Genentech), an antiangiogenic MAb targeting VEGF, is approved for the treatment of colorectal cancer. To fully realize the potential of targeting endothelial cells, it may be necessary to exploit new or yet to be identified targets of the tumor vasculature. Recently identified tumor endothelial markers may provide the molecular specificity needed to exploit this approach, and such molecules are likely to play important roles in the regulation of angiogenesis.

15.11.21.1 Alpha-Emitting Antibodies for Vascular Targeting

Alpha particles are extraordinarily potent, short-ranged radiations with geometry uniquely suitable for selectively killing neovasculature. Actinium-225 (^{225}Ac)-E4G10, an alpha-emitting antibody construct reactive with the unengaged form of vascular endothelial cadherin, is capable of potent, selective killing of tumor neovascular endothelium and late endothelial progenitors in bone marrow and blood (Jaggi et al. 2007). No specific normal tissue uptake of E4G10 was seen by imaging or postmortem biodistribution studies in mice. In a mouse model of prostatic carcinoma, ^{225}Ac-E4G10 treatment resulted in inhibition of tumor growth, lower serum PSA level, and markedly prolonged survival, which was further enhanced by subsequent administration of paclitaxel. Immunohistochemistry revealed lower vessel density and enhanced tumor cell apoptosis in ^{225}Ac-E4G10-treated tumors. Additionally, the residual tumor vasculature appeared normalized, and no toxicity was observed in vascularized normal organs following ^{225}Ac-E4G10 therapy. The data suggest that alpha-particle immunotherapy to neovasculature, alone or in combination with sequential chemotherapy, is an effective approach to cancer therapy.

15.11.21.2 Angiolytic Therapy

In contrast to antiangiogenesis therapy directed again the developing blood vessels in tumor, angiolytic therapy aims to destroy the existing vasculature of tumors. Usually the central portion of a solid cancer has an inadequate blood supply through its poorly developed network of tortuous and malformed microvessels. This poor vascular supply causes reduced delivery of chemotherapy to the central parts of the tumor mass. Further, tumor hypoxia limits the action of cancer therapies such as radiotherapy, which requires oxygen and an active cell metabolism to be effective. High doses of anticancer therapies are often given in an attempt to overcome these factors and to kill as much of the central part of the tumor as possible. However, because of the low-oxygen environment, this step does not necessarily kill all of the

tumor cells despite the escalated doses. While hypoxia is the basis of some anticancer therapies, angiolytic therapy aims to destroy the abnormal blood vessels.

15.11.21.3 Anti-phosphatidylserine Antibodies as VTA

Phosphatidylserine (PS) is an anionic phospholipid. The main function of phospholipids is the formation of cellular membranes. In normal cells, anionic phospholipids are on the inside of the cellular membrane. Exposure of anionic phospholipids on the cell surface occurs during apoptosis, cell injury, cell activation, and malignant transformation. Factors in the tumor microenvironment cause a breakdown of asymmetry and exposure of anionic phospholipids on the cell surface of the blood vessel and malignant cells.

Anionic phospholipids are attractive as tumor blood vessel targets for several reasons: they are abundant; they are on the surface of the endothelial cells that line tumor vessels that is accessible to VTAs in the blood; they are present on a significant percentage of endothelial cells in diverse solid tumors; and they appear to be absent from vascular endothelium in all normal tissues.

15.11.21.4 Vadimezan

Vadimezan, formerly AS1404, with the chemical name of DMXAA (5,6-dimethylxanthenone-4-acetic acid), is a small-molecule “vascular-targeting agent” that selectively disrupts established tumor blood vessels. The basis for the targeting of tumor blood vessels by ASA404 is thought to lie in the distinctive features of tumor blood vessels: the capillary network is more permeable and less well organized than that of a normal tissue. Both direct and indirect effects on tumor blood vessels have been implicated in the action of vadimezan. Direct action is on the endothelial cells that line tumor blood vessels, causing apoptosis (cell suicide). Vadimezan also acts indirectly, causing the release of von Willebrand factor, which leads to blood clotting and occlusion of blood vessels. In addition, it triggers a local cascade of cytokines (biochemical mediators) including serotonin and tumor necrosis factor. Effects of vadimezan are partly due to it acting as a multikinase inhibitor and the anti-VEGFR activity in particular may contribute to the non-immune-mediated effects of vadimezan on the vasculature (Buchanan et al. 2012). The direct and indirect effects of vadimezan culminate in the breakdown of the vasculature and the death of tumor cells. It has also been combined with chemotherapy for solid cancers.

The therapeutic potential of vadimezan lies in combination with cytotoxic agents and other cancer treatments. Preclinical tests have shown synergistic effects with such combinations, and they have now become the focus for human clinical trials. Results of phase II trials with vadimezan in combination with standard chemotherapy suggested a survival advantage in NSCLC comparable to that achieved with bevacizumab but with little additional toxicity (Head and Jameson 2010). Vadimezan

showed promising effects in phase II clinical trials in NSCLC. However, these effects were not replicated in phase III clinical trials. Novartis is now responsible for further development work on the drug.

15.11.21.5 Cadherin Inhibitors

One of the factors maintaining vascular integrity is VE-cadherin, which is a cell-to-cell adhesion molecule present in the endothelium of blood vessels (like N-cadherin). Antibodies to VE-cadherin have been shown to have profound antiangiogenic and possibly angiolytic effects on blood vessels in tumor-bearing animals. Thus, peptides or organic molecules that have an antagonistic activity at VE-cadherin receptors might have interesting vascular effects that could be exploitable therapeutically in cancer.

ADH-1 (Exherin™, Adherex Technologies), a small peptide molecule developed by rational drug design, competitively inhibits N-cadherins that hold the tumor blood vessels together and selectively ruptures the blood vessels in cancers sparing healthy blood vessels outside the tumor. Exherin is infused to strangle and destroy as much of a tumor as possible, using the tumor's dysfunctional vasculature as a way to cut off all effective blood flow and killing the cancer cells in the tumor interior. Conventional chemotherapy or radiation can then be employed to eradicate the peripheral, surviving islands of the tumor. Within 30–60 min after infusion of ADH-1, tumor blood flow virtually ceases. A phase I study showed that ADH-1 is a well-tolerated drug with a modest anticancer effect in tumors which express N-cadherin (Yarom et al. 2013). It is currently in phase II clinical trials. If a chemotherapeutic drug is given prior to Exherin™, there could be three interrelated outcomes:

- The chemotherapeutic may leak from the damaged intratumoral blood vessels flooding high concentrations of the chemotherapeutic into the tumor tissue.
- The subsequent vascular shutdown in the tumor induced by Exherin™ would then lock in the chemotherapeutic within the tumor, with no vascular flow left to drain the drug from the tumor. This locked-in chemotherapeutic drug would then have a prolonged residency in the tumor and hence prolonged activity at the tumor site.
- This ability to concentrate drug at the tumor site could then lead to reduction of exposure to the anticancer agents elsewhere in the body and hence reduced side effects.

15.11.21.6 Fosbretabulin Tromethamine

Fosbretabulin tromethamine, or combretastatin A4 prodrug (CA4P, OXiGENE's ZYBRESTAT), is a synthetic vascular-targeting agent. It was originally derived from the root bark of the *Combretum caffrum* tree, also known as the Cape Bushwillow. Combretastatin is introduced into a patient's bloodstream using a conventional intravenous infusion. After infusion, the soluble prodrug rapidly

distributes throughout the patient's bloodstream. Enzymes in the patient's blood convert the water-soluble prodrug into an active form of the prodrug. The active form of prodrug quickly enters the endothelial cells lining the blood vessels. Endothelial cells that line tumor-associated blood vessels are particularly sensitive to combretastatin's effects because they are immature endothelial cells as compared to the more mature cells that line blood vessels in normal tissues. It is the immaturity of these cells that CA4P exploits. Once inside the immature endothelial cell, CA4P is able to disrupt the internal skeleton that gives the endothelial cell its characteristic flat shape (CA4P does not do this to mature endothelial cells because they are protected by Actin, which is not fully present in immature endothelial cells). Without an internal skeleton to maintain their elongated shape, the endothelial cells change from a flattened streamlined profile to a rounded, bloated profile. The bloated endothelial cells effectively plug the capillaries and prevent the blood flow necessary to feed the patient's tumor. In a phase I study, the toxicity profile was consistent with a drug that is "vascularly active" and devoid of traditional "cytotoxic" side effects. Phase I/II clinical trials have shown ZYBRESTAT's ability to reduce blood flow to the tumor measured by MRI, thus inhibiting the tumor's survival and growth. The studies were the first demonstrations in human clinical trials of an inhibitor that blocks the flow of blood within tumor-associated blood vessels. ZYBRESTAT has shown encouraging results in a phase II study of patients with anaplastic thyroid cancer and is in phase II trials for relapsed ovarian cancer.

15.11.21.7 Vascular-Targeting Agents Versus Antiangiogenesis Agents

Both antiangiogenesis and vascular-targeting approaches to the treatment of cancer fit very well with conventional anticancer therapy, and combined therapies are expected to be more effective than any individual method.

Antiangiogenesis agents prevent vascular endothelial cell division while having little or no effect on vasculature where division is not taking place. They appear to inhibit tumor growth in regions of neovascularization but do not prevent growth of tumors along existing vascular tracts or survival of tumor cells in regions served by mature, nonproliferating vessels. Therefore, antiangiogenesis agents are most effective against tumors and metastases where angiogenesis is occurring vigorously and less effective against large tumors with a more established vasculature. Antiangiogenesis agents must be administered for prolonged periods to continue to suppress tumor growth.

15.11.22 Delivery of Proteins and Peptides for Cancer Therapy

Several drugs for the treatment of cancer are proteins and need to be given by subcutaneous injection. These agents include cytokines, MAbs, and interferons. The potential of peptide and protein anticancer agents has yet to be realized owing

to the many unresolved problems concerning their delivery to the site of a tumor and into tumor cells. Objections to the delivery of proteins and peptides by injection are as follows:

- It is painful, and multiple daily injections present a compliance problem.
- Injectable proteins have strict storage requirements and can suffer loss of activity.
- Hypodermic needle disposal is a problem.
- Protein drugs are liable to adhere to the walls of the vial and the syringe.

Problems with proteins and peptides, which are relevant to drug delivery, are as follows:

- They are large molecules with complex structure.
- They are liable to be taken up by nontarget organs and tissues.
- Fast elimination from the systemic circulation because of degradation and renal clearance.
- They are liable to induce an immune reaction.
- They are unstable in natural environments and are liable to undergo proteolysis.
- They are expensive to produce.

However, our understanding of the mechanisms underlying the biological fate and biodistribution of protein and peptide drugs has advanced to the stage where methods that use or influence these mechanisms are now available. There are different approaches that can improve the stability, longevity, and targeting of peptides and proteins in the body. Efforts are being made to find formulations for alternative non-injection routes, such as oral delivery. Examples of protein and peptide anticancer drugs that are under investigation for alternative routes of delivery are as follows:

- Inovio Pharmaceuticals' CELLECTRA™ for DNA delivery by electroporation.
- Emisphere's Eligen™ system for oral administration of protein drugs.
- Nasally delivered leuprolide, an analog of LHRH, is marketed for prostate cancer.
- Modification of proteins and peptides with polymers.

15.11.22.1 CELLECTRA™ Electroporation Device

CELLECTRA™ electroporation (Inovio Pharmaceuticals) uses controlled millisecond electrical pulses to create temporary pores in the cell membrane and enable cellular uptake of a synthetic DNA vaccine previously injected into muscle or skin. The cellular machinery then uses the DNA's instructions to produce one or more proteins associated with the targeted disease. These foreign proteins or antigens mimic the presence of an actual pathogen and induce an immune response to provide protection against the pathogen or eliminate cells that have undergone malignant change. CELLECTRA™ is used to deliver Inovio's SynCon® vaccines to treat several cancers, including cervical cancer (in phase II clinical trials) and prostate cancer (phase I clinical trials). Inovio is collaborating on DNA vaccine programs to treat AML and CML and multiple cancers that express the hTERT antigen.

15.11.22.2 Emisphere's Eligen™ System

Emisphere delivery agents utilize the body's natural passive transcellular transport process, allowing macromolecules to cross membranes and yet remain therapeutically active. Studies have shown that Emisphere's technology does not affect the intestinal membrane in this process. Thus, oral delivery using the Eligen™ system occurs without chemical modification of the molecule or damage to the biological membrane. Emisphere delivery agents enable molecules that are too large or too charged to cross cell membranes. Once the drug crosses the membrane, the delivery agent dissociates from the drug, and the drug reestablishes its natural distribution of conformations, ensuring that the delivered drug is in its therapeutically active state. Advantages of this technology include the following:

- Broad applicability.
- It is applicable across a diverse group of drug molecules (e.g., proteins, polysaccharides, peptides, and other poorly absorbed compounds).
- It is a stand-alone delivery approach.
- Oral drug delivery using an Emisphere delivery agent does not rely upon the addition of other agents that can have adverse effects on the intestinal membranes or digestion process (e.g., penetration enhancers or enzyme inhibitors).
- Versatility of formulation. Emisphere has created various types of oral formulations, including solutions, suspensions, tablets, and capsules. Emisphere also believes its oral drug delivery technology is applicable to controlled release dosage forms.
- Ease of manufacture. The technology and manufacturing equipment required to produce Emisphere delivery agent material in commercial quantities is readily available.

15.11.22.3 Diatos Peptide Vector Intracellular/Intranuclear Delivery Technology

Diatos peptide vector (DPV) involves direct intracellular delivery using cell-penetrating peptides and enables the release of anticancer agents selectively at the sites of tumor cells. The features of this technology are as follows:

- 15–20 amino acid polycationic peptides derived from human anti-DNA antibodies and human heparin-binding proteins
- Chemically or genetically linked to the therapeutic molecule
- Bind to cell surface glycosaminoglycans and penetrate via energy-dependent endocytosis
- Provide access to previously inaccessible intracytoplasmic or intranuclear targets
- Selectively deliver small or large molecules, including therapeutic proteins or peptides, antibodies or antibody fragments, oligonucleotides, and nanospheres into the cytosol or the nucleus

15.11.22.4 Modification of Proteins and Peptides with Polymers

In some cases, the circulation time of drugs can be increased by conjugating them with polymers that are not large enough to prevent renal clearance themselves, but which can attach themselves, along with their conjugated drug, to natural long-circulating blood plasma components, such as serum albumin or lipoproteins. An example of such a polymer is poly(styrene-co-maleic acid anhydride) (SMA). Conjugation of peptides and proteins with this 1.5-kDa polymer increases the circulation time of anticancer proteins and peptides because the conjugates bind to plasma albumin. As with the conjugation of drugs with high-MW polymers, conjugation with SMA protects proteins from enzymatic degradation and decreases the immunogenicity of the modified proteins. At least one protein-based SMA-modified pharmaceutical, neocarzinostatin–SMA conjugate is currently approved in Japan for treating hepatoma. Several other anticancer formulations based on anticancer peptides and proteins modified with SMA have shown promising results in preclinical studies and clinical trials. Advantages of peptide and protein–polymer conjugates are as follows:

- Decreased renal filtration
- Protection against enzymatic degradation
- Lowered immunogenicity
- Selective accumulation in tumors via the EPR effect

Other methods include incorporation into microparticulate drug carriers, EPR effect-based tumor targeting, and the use of targeting moieties. Furthermore, new approaches to intracellular drug delivery, including the use of transduction proteins and peptides, are being developed.

15.11.22.5 Peptide-Based Targeting of Cancer Biomarkers for Drug Delivery

Small peptide-based agents attracted wide interest as cancer-targeting agents for diagnostic imaging and targeted therapy. Efforts were made to develop high-affinity and high-specificity peptidomimetic or small-molecule ligands against cancer cell surface receptors. A high-affinity peptidomimetic ligand (LLP2A) against α4β1 integrin has been identified using both diverse and highly focused one-bead, one-compound combinatorial peptidomimetic libraries in conjunction with high-stringency screening (Peng et al. 2006). LLP2A can be used to image α4β1-expressing lymphomas with high sensitivity and specificity when conjugated to a near-infrared fluorescent dye in a mouse xenograft model. Thus, LLP2A provides an important tool for noninvasive monitoring of α4β1 expression and activity during tumor progression, and it shows great potential as an imaging as well as therapeutic agent delivery for α4β1-positive tumors.

Although MAbs to CD20, e.g., rituximab, also target low-grade B-cell lymphoma, their limitations are as follows: (1) they do not bind to T cells and are, therefore, ineffective in the treatment of T-cell lymphoma; (2) they bind to the reticuloendothelial system with high liver and spleen uptake; (3) and anti-CD20 MAbs

also bind to normal B lymphocytes. In contrast to LLP2A, ligand binds to both B- and T-cell cancer lines, has low uptake in the liver and spleen, and has low affinity for normal B and T lymphocytes.

15.11.22.6 Peptide–Cytokine Complexes as Vascular-Targeting Agents

NGR-hTNFα (MolMed S.p.A.'s ARENEGYR) is a novel vascular-targeting agent with a unique mode of action and a first-in-class compound in the class of peptide–cytokine complexes able to selectively target the tumor vasculature. ARENEGYR consists of a tumor-homing peptide (NGR) selectively binding tumor blood vessels, fused to the powerful anticancer cytokine hTNFα. The resulting molecule has unique biological properties, including a direct biological antitumor activity and induction of tumor vascular permeability and normalization, thereby improving the therapeutic efficacy of cytotoxic drugs administered in combination. This makes NGR-hTNFα particularly attractive both as single agent and as part of combination therapy with several chemotherapeutic regimens. Moreover, NGR-hTNFα has shown a very favorable safety profile so far and appears to act independently of tumor type with low risk of inducing pharmacological resistance and therefore has potential applications in the treatment of most solid tumors.

NGR-hTNFα is undergoing clinical development both as single agent and in combination with several different chemotherapeutic agents. Phase II trials of NGR-hTNFα as single agent are ongoing in colorectal and liver cancer and in mesothelioma. Ongoing combination trials include phase II with XELOX in colorectal cancer and with doxorubicin in SCLC (after treatment of a first sample size in the single-agent setting) and a phase I trial in combination with cisplatin. NGR-hTNFα has orphan drug designation in the European Union for the treatment of malignant mesothelioma.

15.11.22.7 Peptide–Polymer Conjugates with Radionuclides

A binding interaction between Mucin1 (MUC1) peptide and a single-chain variable fragment (scFv) antibody was selected from a scFv library screened against MUC1 for the design of molecules used for targeted delivery of radioimmunotherapeutic agents for prostate and breast cancer treatment (Sulchek et al. 2005). Dynamic force spectroscopy was used to estimate the intermolecular potential widths and equivalent thermodynamic off rates for monovalent, bivalent, and trivalent interactions. The results demonstrate that an increase of the interaction valency leads to a precipitous decline in the dissociation rate. Binding forces measured for monovalent and multivalent interactions match the predictions of a Markovian model for the strength of multiple uncorrelated bonds in a parallel configuration. This approach is promising for comparison of the specific effects of molecular modifications as well as for determination of the best configuration of antibody-based multivalent targeting agents. Such cancer-targeting agents, when injected into the blood, not only bind to tumor cells but also efficiently catch a subsequently administered small radioactive molecule, resulting in greatly enhanced tumor-targeted radiotherapy.

15.11.23 Image-Guided Personalized Drug Delivery in Cancer

Image-guided drug delivery (IGDD) in cancer is a form of individualized therapy where imaging methods are used in guidance and monitoring of localized and targeted delivery of therapeutics to the tumor. A systematic approach to IGDD requires mechanisms for targeting, delivery, activation, and monitoring of the process. Although the goal in IGDD is to optimize the therapeutic ratio through personalized image-guided treatments, a major challenge is in overcoming the biological barriers to the delivery of therapeutics into tumors and cells. Physiologic and quantitative imaging techniques may serve as enabling tools that could potentially transform many existing challenges into opportunities for advancement of the field (Tandon and Farahani 2011).

15.11.24 A Computational Approach to Integration of Drug Delivery Methods for Cancer

The advent of sophisticated drug delivery strategies for cancer applications has flooded the scientific and clinical community with new tactics and approaches such as molecular targeting and nanotechnology-based methods. Unfortunately, the clinical impact has been moderate at best, falling significantly short of revolutionizing existing chemotherapeutic methods. Currently, a cancer patient has a higher probability of receiving traditional systemically administered drugs than a more sophisticated targeted or nanotechnology-based therapeutic. One approach acknowledges the significance of the numerous biological barriers presented in the human body and their sequential nature. This approach recommends that computational mathematical tools should be used to predict which nanovectors, surface modifications, therapeutic agents, and penetration enhancers to use for a multistage drug delivery strategy (Sakamoto et al. 2007). Several stages of micro-/nanovectors are nested within each other and delivered to overcome specific biological barriers to ultimately release a concentrated dose of a therapeutic payload at the intended lesion site. This novel, multistage strategy enables efficient localized delivery of chemotherapeutic drugs that may lead to significant improvements in therapy efficacy, reduced systemic toxicity, and decrease in total amount of injected drugs.

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Chapter 16
Personalized Cancer Therapy

16.1 Introduction

Individual variation in response to a therapy for the same type of cancer is well known; some patients show no response, whereas others show a dramatic response. It is obvious that the concept "one medicine for all patients with the same disease" does not hold and a more individualized approach is needed. Although individualization of certain treatments has been carried out in the pregenomic era, the concept of personalized medicine as described in this chapter follows progress in the study of human diseases at the molecular level, advances in molecular diagnostics, and drug development based on various omics such as genomics, proteomics, and metabolomics and biomarkers. The aim of the personalized medicine is to match the right drug to the right patient. The broad scope of personalized medicine includes predisposition testing, determination of prognosis, combination of diagnostics with therapeutics, and monitoring of therapy.

There is no officially recognized definition of personalized medicine. The term "personalized medicine" was first used as the title of a monograph in 1998 (Jain 1998) and started to appear in MEDLINE in 1999, but most of the literature relevant to personalized medicine continued to be indexed under pharmacogenomics and pharmacogenetics (Jain 2001, 2002). Personalized medicine simply means the prescription of specific treatments and therapeutics best suited for an individual taking into consideration both genetic and environmental factors that influence response to therapy. Personalized medicine is the best way to integrate new biotechnologies into medicine for improving the understanding of pathomechanism of diseases and management of patients (Jain 2009a).

Management of cancer has been unsatisfactory in the past, but an understanding of the molecular, genetic, and genomic aspects of cancer is accelerating progress in personalized cancer therapy. Several comprehensive studies have demonstrated the utility of gene expression profiles for the classification of tumors into clinically relevant subtypes and the prediction of clinical outcomes. The role of oncoproteomics in personalized management of cancer was described in Chap. 4.

K.K. Jain, *Applications of Biotechnology in Oncology*, DOI 10.1007/978-1-4614-9245-0_16,
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Table 16.1 Factors that drive the development of personalized therapy in cancer

Advances in application of proteomic technologies in cancer
Advances in cancer vaccine technologies
Cancer biomarkers can be used for diagnosis as well as drug targets
Examples of personalized treatment of cancer are already in practice
Incentive to development from motivated physicians, patients, and third-party payers
Increasing cancer burden with aging US population is a driving force for development. At current incidence rates, the total number of cancer cases is expected to double by 2050 (1.3–2.6 million)
Molecular diagnosis of cancer is advancing rapidly
Progress in pathophysiology of cancer
Search for better treatments due to limited efficacy and toxicity of chemotherapy
Sequencing is increasingly applied to understanding cancer and molecular diagnosis
Transcriptional profiling in cancer

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Considerable progress has been made in this field during the past few years. Factors that drive the development of personalized therapy for cancer are listed in Table 16.1. Earlier chapters have described cancer cell therapy and cancer vaccines, and information presented in this section will show personalization of these therapies.

16.1.1 Challenges of Cancer Classification

Cancer is a very heterogeneous disease. Current classifications of cancer are based on the type of tissue of origin, histological appearance, and tendency to metastasize. These provide only a limited view of cancer. It is now known that cancer varies both genetically and phenotypically between patients who may have the identical type and stage of cancer. Each person's cancer is as unique as his or her fingerprint. This variability helps to explain unpredictable responses to existing drug therapies that have been observed to date. Large-scale expression monitoring on microarrays has provided the ability to look at cancer at a molecular level and transcription of messenger RNA (mRNA) messages from genes—transcriptional profiling.

Tumor heterogeneity is underestimated as it is not heterogeneity between tumors, but heterogeneity within an individual tumor as well, which has been mapped out (Gerlinger et al. 2012). Multiple samples from each patient's primary and metastatic tumor sites were obtained in a study of renal cell cancer before and after treatment. About two-thirds of the mutations that were found in single biopsies were not uniformly detectable throughout all the sampled regions of the same patient's tumor. A "favorable prognosis" gene profile and an "unfavorable prognosis" gene profile were expressed in different regions of the same tumor. Therefore, a single tumor biopsy cannot be considered representative of the landscape of genomic abnormalities in a tumor. Another finding of this is that different regions of the tumor have different mutations in the very same genes (the so-called convergent evolution), including in SETD2, PTEN, and KDM5C, which underscores the importance of

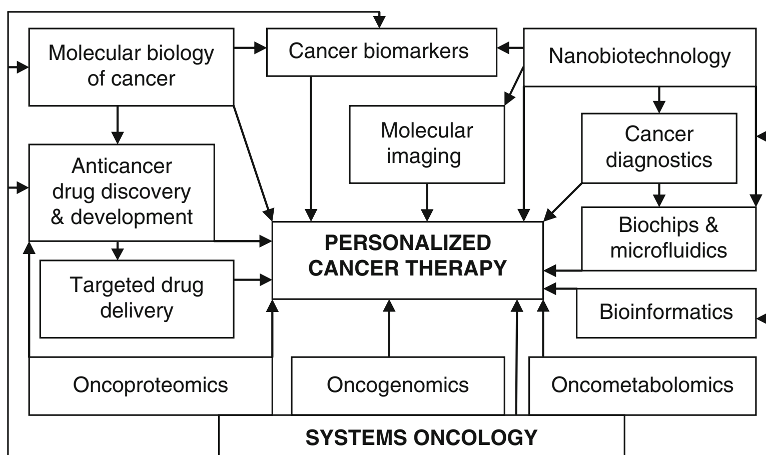

Fig. 16.1 Relationships of technologies for personalized management of cancer

changing particular tumor cell functions as the tumor expands and evolves. From the function of the genes that were targeted for different mutations, it would appear that alterations in epigenetic mechanisms and signal transduction as the tumor evolves are keys to the tumor's survival. Genes that are affected by convergent evolution may be suitable targets for functional inhibition or restoration.

16.1.2 Relationships of Technologies for Personalized Management of Cancer

Cancer is a good example of integration of various technologies for personalized management as shown in Fig. 16.1.

The biggest challenge for optimal treatment outcomes in cancer patients is the complex nature of the disease due to cellular heterogeneity and dysfunction of numerous molecular networks as results of genetic as well as environmental disturbances. A disease with multifactorial origin such as cancer requires a multipronged attack. For increasing anticancer therapeutic efficacy, one should attack all the oncogenic nodes.

16.1.3 Systems Biology Approach to Cancer

Systems biology, with its holistic approach to understanding fundamental principles in biology, and the empowering technologies in genomics, proteomics, single-cell

analysis, microfluidics, and computational strategies, enables a comprehensive approach to cancer with an attempt to unveil the pathogenic mechanisms of diseases, identify disease biomarkers, and provide new strategies for drug target discovery. Integration of multidimensional high-throughput "omics" measurements from tumor tissues and corresponding blood specimens, together with new systems strategies for diagnostics, enables the identification of cancer biomarkers that can enable presymptomatic diagnosis, stratification of disease, assessment of disease progression, evaluation of patient response to therapy, and the identification of recurrences. Although some aspects of systems medicine are being adopted in clinical oncology practice through companion molecular diagnostics for personalized therapy, the increasing amount of global quantitative data from both healthy and diseased states is shaping up a transformational paradigm in medicine that is termed "predictive," "preventive," "personalized," and "participatory" (P4) medicine, which requires new scientific and organizational strategies to translate this approach to the healthcare system (Tian et al. 2012).

16.2 Impact of Molecular Diagnostics on the Management of Cancer

Molecular diagnosis influences cancer management in several ways that lead to personalization (Table 16.2). These technologies are enabling the classification of cancer based on molecular profiles as a basis for more effective personalized therapies. Various tests have been used to predict response to treatment and prognosis.

16.2.1 Analysis of RNA Splicing Events in Cancer

Alternative splicing has a role in several aspects of cancer treatment, including the failure of the patient to activate the administered drug, high toxicity owing to inappropriate metabolism, and variability of the apoptotic thresholds necessary to trigger cell death. Genetic variations within both the patient and the tumor cause changes in the apoptotic threshold and thus differences in both the toxicity and the efficacy of a chemotherapy drug. Differential expression of a large number of apoptotic alternative RNA splice variants is well documented in tumors and shows a correlation with drug response. Antisense approaches can target specific antiapoptotic splice variants to lower the apoptotic threshold of a tumor cell and therefore increase the efficacy of chemotherapy drugs. As RNA splicing is deregulated in human cancers, it is likely that such alterations will provide pharmacogenomically relevant biomarkers. Gene expression profiling (GEP) technologies such as DATA (differential analysis of transcripts with alternative splicing) could be applied to identify RNA splicing differences between tumor biopsies that respond to treatment compared with those that do not respond.

Table 16.2 Impact of molecular diagnostics on personalized management of cancer

Classification of cancer
Analysis of RNA splicing events in cancer
Cancer classification using microarrays
Cancer stratification based on methylation markers
Characteristic of circulating cancer cells
eTag assay system for cancer biomarkers
Gene expression profiling
Risk assessment and prognosis
Cancer prognosis
Detection of mutations for risk assessment and prevention
Prediction of response to treatment
Biopsy testing of tumors for chemotherapy sensitivity
Genomic analysis of tumor biopsies to predict response to treatment
Prediction of response to radiation therapy
Serum nucleosomes as indicators of sensitivity to chemotherapy
Testing microsatellite-instability for response to chemotherapy
Diagnostics as guide to therapeutics
Diagnostics for detection of minimal residual disease
Detection of resistance to chemotherapy
Molecular diagnostics combined with cancer therapeutics
Drug discovery and development
Design of future cancer therapies
Screening for personalized anticancer drugs
Pharmacogenomic tests for stratification of clinical trials

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16.2.2 Analysis of Chromosomal Alterations in Cancer Cells

Cancer cells have a remarkable ability to disable some genes and overuse others, allowing their unchecked growth into tumors. The most aggressive of these distortions occurs when cells delete or multiply chunks of their own chromosomes. Cells can simply snip strings of genes from the chromosome or make many extra copies of the string and reinsert it into the chromosome. Fast and reliable methods are available for identifying alterations to chromosomes that occur when cells become malignant. Data can be analyzed by advanced statistical techniques to accurately detect deletions and additions. Many previously unknown additions and deletions were found in human breast cancer cells. The technique helps to show how cells modify their own genetic makeup and may allow cancer treatments to be tailored more precisely to a patient's disease.

16.2.3 Cancer Classification Using Microarrays

Classification of a cancer based on gene expression profile is important for personalizing cancer therapy. In the process of expression profiling, robotically printed DNA microarrays are used to measure the expression of tens of thousands of genes at a

time; this creates a molecular profile of the RNA in a tumor sample. A variety of analytic techniques are used to classify cancers on the basis of their gene expression profiles. There are two general approaches. In an unsupervised approach, pattern recognition algorithms are used to identify subgroups of tumors that have related gene expression profiles. In a supervised approach, statistical methods are used to relate gene expression data and clinical data. The determination of tumor marker genes from gene expression data requires bioinformatic tools because expression levels of many genes are not measurably affected by carcinogenic changes in the cells. These molecular markers give valuable additional information for tumor diagnosis/prognosis and will be important for the development of personalized therapy of cancer.

An example of the application of microarrays for gene expression is bladder cancer, a common malignant disease characterized by frequent recurrences. The stage of disease at diagnosis and the presence of surrounding carcinoma in situ are important in determining the disease course of an affected individual. Clinically relevant subclasses of some cancers have been identified using expression microarray analysis of well-characterized tumors. It is possible to classify benign and muscle-invasive tumors with close correlation to pathological staging and better predictive information on disease progression as compared with conventional staging. Furthermore, gene expression profiles characterizing each stage and subtype identified their biological properties, producing new potential targets for therapy.

Gene expression microarray technology is helpful in all phases of the discovery, development, and subsequent use of new cancer therapeutics, e.g., the identification of potential targets for molecular therapeutics. It can be used to identify molecular biomarkers for proof-of-concept studies, pharmacodynamic endpoints, and prognostic markers for predicting outcome and patient selection. Expression profiling can be used alongside gene knockout or knockdown methods such as RNA interference (RNAi).

16.2.4 Diagnosis of Cancer of an Unknown Primary Site

Metastatic cancer of unknown primary site (CUP) accounts for approximately 3 % of all malignant neoplasms and is therefore one of the ten most frequent cancer diagnoses in humans. Patients with CUP present with metastatic disease for which the site of origin cannot be identified at the time of diagnosis. It is now accepted that CUP represents a heterogeneous group of malignancies that share a unique clinical behavior and, presumably, unique biology. Extensive work-up with specific pathology investigations (immunohistochemistry (IHC), electron microscopy, molecular diagnosis) and modern imaging technology (CT, mammography, positron emission tomography (PET) scan) have resulted in some improvements in diagnosis, but the primary site remains unknown in most patients. The most frequently detected primaries are carcinomas hidden in the lung or pancreas. Several favorable subsets of CUP have been identified, which are responsive to systemic chemotherapy and/or

locoregional treatment. Identification and treatment of these patients are important. The considered responsive subsets to platinum-based chemotherapy (PBC) are the poorly differentiated carcinomas involving the mediastinal–retroperitoneal nodes, the peritoneal papillary serous adenocarcinomatosis in females, and the poorly differentiated neuroendocrine carcinomas. Other tumors successfully managed by locoregional treatment with surgery and/or irradiation are the metastatic adenocarcinoma of isolated axillary nodes, metastatic squamous cell carcinoma of cervical nodes, or any other single metastatic site. Diagnosis of CUP is important for personalized management of cancer. Pathwork Informatics Inc. is developing diagnostics for CUP using microarrays and gene expression analysis.

16.2.5 DNA Repair Biomarkers

Most chemotherapeutics and radiation therapy work by damaging DNA. All solid cancers have multiple pathways and some of these can enable tumor cells to survive DNA damage induced by chemotherapeutic treatments. Therefore, inhibitors of specific DNA repair pathways might prove effective when used in combination with DNA-damaging chemotherapeutic drugs. In addition, alterations in DNA repair pathways that arise during tumor development can make some cancer cells reliant on a reduced set of DNA repair pathways for survival. There is evidence that drugs that inhibit one of these pathways in such tumors could prove useful as single-agent therapies, with the potential advantage that this approach could be selective for tumor cells and have fewer side effects (Helleday et al. 2008). Proteomic biomarkers in each of the pathways differ according to the type of cancer, but they overlap. Since most available cancer therapeutics work by inducing DNA damage, which causes cell death, monitoring specific DNA repair biomarkers may indicate whether the therapeutic is producing tumor cell death. DNA repair biomarkers could enable physicians to monitor treatment effectiveness from solid tumor samples.

16.2.6 Gene Expression Profiling

GEP has been used to improve the design of cancer drugs that have shown some promise in clinical trials. Microarray methods have revealed unexpected subgroups within the diagnostic categories of the hematologic cancers that are based on morphology and have demonstrated that the response to therapy is dictated by multiple independent biologic features of a tumor. Some applications of this approach are:

- These expression signatures can be combined to form a multivariate predictor of survival after chemotherapy for diffuse large-B-cell lymphoma.
- GEP has been used as an alternative approach to mapping chromosomal translocations in leukemias. Gene expression signatures can be combined with the use of statistical algorithms to predict chromosomal abnormalities with a high degree of accuracy.

- In B-cell acute lymphoblastic leukemia (ALL), GEP at the time of diagnosis provides information that could predict which patients would relapse and which would remain in continuous complete remission.

An important goal is to develop a platform for routine clinical diagnosis that can quantitatively measure the expression of a few hundred genes. Such a diagnostic platform would enable a quick determination of important molecular subgroups within each hematologic cancer. As new clinical trials designed, one must include genomic-scale GEP in order to identify the genes that influence the response to the agents under investigation. Thus the molecular diagnosis of the hematologic cancers can be refined on the basis of new advances in treatment and facilitate the development of tailored therapies for molecularly defined diseases.

GEP has been done of prostate tumors using IHC on tissue microarrays. Positive staining for MUC1, a gene highly expressed in the subgroups with aggressive clinicopathological features, is associated with an elevated risk of recurrence, whereas strong staining for AZGP1, a gene highly expressed in the other subgroup, is associated with a decreased risk of recurrence. In multivariate analysis, MUC1 and AZGP1 staining were strong predictors of tumor recurrence independent of tumor grade, stage, and preoperative prostate-specific antigen (PSA) levels. These observations suggest that prostate tumors can be usefully classified according to their gene expression patterns, and these tumor subtypes may provide a basis for improved stratification for prognosis and treatment.

Some of the cancer signatures can predict clinical response in individuals treated with anticancer drugs. Notably, signatures developed to predict response to individual agents, when combined, could also predict response to multidrug regimens. Finally, integration of chemotherapy response signatures with signatures of oncogenic pathway deregulation may help to identify new therapeutic strategies that make use of all available drugs. The development of gene expression profiles that can predict response to commonly used cytotoxic agents provides opportunities to better use these drugs, including their use in combination with existing targeted therapies.

16.2.6.1 OnkoMatch Tumor Genotyping

OnkoMatch™ tumor genotyping (GenPath Oncology), based on polymerase chain reaction (PCR) amplification followed by single base extension detection of hotspot mutations that have been identified as key driver mutations, provides a reliable and robust tumor genotyping platform for detecting 68 mutations (including epidermal growth factor receptor (EGFR), BRAF, and K-ras) across 14 oncogenes. It was developed at the Massachusetts General Hospital where SNaPshot Multiplex System has been applied for genotyping tumors such as non-small-cell lung cancer (NSCLC) and in influencing treatment decisions as well as directing patients toward relevant clinical trials (Sequist et al. 2011).

16.2.6.2 Gene Expression Profiles Predict Chromosomal Instability in Tumors

Microscopic examination of tumor specimens cannot always predict a cancer's aggressiveness, leading to increased interest in molecular approaches to diagnosis. A genetic profile indicating chromosomal instability (CIN)—an increased tendency to develop chromosomal aberrations that are critical in cancer development—is predictive of clinical outcome in a broad range of cancer types (Carter et al. 2006). The findings and conclusions of this study were as follows.

CIN leads to a condition known as aneuploidy, in which chunks of DNA are either missing or duplicated. The technique indirectly measures the degree of aneuploidy and thus the degree of CIN by looking for abnormal expression levels of genes at the different chromosomal locations. The authors identified a 25-gene signature of CIN from specific genes whose expression was consistently correlated with total functional aneuploidy in several cancer types. This signature was a significant predictor of clinical outcomes in a variety of cancers (breast, lung, medulloblastoma, glioma, mesothelioma, and lymphoma). It could also differentiate between primary tumors and tumor metastases and, in grade 1 and grade 2 breast cancers, distinguished the more aggressive cancer within each grade. Using gene expression data from 18 previous studies of cancer, representing 6 cancer types, they found that this genetic profile, or signature, predicted poor clinical outcome in 12 of the populations studied. The technique may form the basis of a diagnostic tool that could be used in the clinic and also help in the search for cancer drugs that reduce CIN. This approach would be useful for developing personalized therapy of cancer.

16.2.7 Detection of Circulating Tumor Cells for Personalized Management of Cancer

Viable tumor-derived epithelial cells (circulating tumor cells or CTCs) have been identified in peripheral blood from cancer patients and are probably the origin of intractable metastatic disease. CTCs represent a potential alternative to invasive biopsies as a source of tumor tissue for the detection, characterization, and monitoring of non-hematologic cancers. Given the high sensitivity and specificity of the CTC-Chip (see Chap. 5), its potential utility has been tested in monitoring response to anticancer therapy. In patients with metastatic cancer undergoing systemic treatment, temporal changes in CTC numbers correlate reasonably well with the clinical course of disease as measured by standard radiographic methods. Thus, the CTC-Chip provides a new and effective tool for accurate identification and measurement of CTCs in patients with cancer. It has broad implications in advancing both cancer biology research and clinical cancer management, including the detection, diagnosis, and monitoring of cancer (Sequist et al. 2009). CTC-Chip has been applied for the personalized management of NSCLC.

16.2.8 Modulation of CYP450 Activity for Cancer Therapy

Metabolism mediated by cytochrome P450 isoenzymes is known to play a major part in the biotransformation of anticancer agents in vivo. Variability between individuals in the pharmacokinetics of anticancer chemotherapeutic agents has an impact on therapeutic efficacy and safety. Since most anticancer agents are transformed by enzymes, a better knowledge of the biotransformation pathways of cyclophosphamide (CPM), ifosfamide, tamoxifen, docetaxel, paclitaxel, and irinotecan could help improve treatment outcome. Furthermore, a better understanding of the metabolism of anticancer agents through phenotyping and genotyping approaches will facilitate the prediction of interactions between drugs. More clinical evidence is needed on the metabolic transformation and drug interactions with these agents to improve cancer therapeutics.

16.2.9 Pathway-Based Analysis of Cancer

16.2.9.1 Conversion of Gene-Level Information into Pathway-Level Information

Gene-level information obtained by gene expression studies needs to be converted into pathway-level information to generate biologically relevant representation of each tumor sample. An algorithm, Pathifier, infers pathway deregulation scores for each tumor sample on the basis of expression data in a context-specific manner for every particular dataset and type of cancer that is being investigated (Drier et al. 2013). Multiple pathway-based representation of algorithm on three colorectal cancer (CRC) datasets as well as two glioblastoma multiforme (GBM) datasets was shown to be reproducible, preserved much of the original information, and enabled inference of complex biologically significant information. They discovered several pathways that were significantly associated with survival of GBM patients and two whose scores are predictive of survival in CRC: CXCR3-mediated signaling and oxidative phosphorylation. They also identified a subclass of proneural and neural GBM with significantly better survival and an EGF receptor-deregulated subclass of CRC. Pathifier is useful for personalized management of cancer.

16.2.9.2 Personalized Therapies Based on Oncogenic Pathway Signatures

The ability to define cancer subtypes, recurrence of disease, and response to specific therapies using DNA microarray-based gene expression signatures has been demonstrated in several studies. By introducing a series of oncogenes into otherwise normal cells and comparing gene expression patterns in normal cells versus cells harboring oncogenes, it can be shown that each cellular signaling pathway is associated with a unique gene expression signature. When evaluated in several large collections of human cancers, these gene expression signatures identify patterns of

pathway deregulation in tumors and clinically relevant associations with disease outcomes. Combining signature-based predictions across several pathways identifies coordinated patterns of pathway deregulation that distinguish between specific cancers and tumor subtypes. The majority of adenocarcinomas of the lung are found to be deregulated for the oncogene Ras, while only a tiny minority of squamous cell carcinomas exhibited Ras deregulation. Hence, deregulation of the Ras pathway is an important signature of adenocarcinomas but not of squamous cell carcinoma.

Clustering tumors based on pathway signatures further defines prognosis in respective patient subsets, demonstrating that patterns of oncogenic pathway deregulation underlie the development of the oncogenic phenotype and reflect the biology and outcome of specific cancers. Predictions of pathway deregulation in cancer cell lines are also shown to predict the sensitivity to therapeutic agents that target components of the pathway. Linking pathway deregulation with sensitivity to therapeutics that target components of the pathway provides an opportunity to make use of these oncogenic pathway signatures to guide the use of personalized cancer therapies. If the Ras and myc pathways are activated in a tumor, physicians could choose drugs that target only myc and Ras. If the SRC and E2F3 pathways are highly active, then drugs can be selected that target these pathways. Because tumors arise from multiple defective genes and their malfunctioning proteins, treatments must target multiple genes and their pathways. The likelihood that someone will be cured by a single drug is low, and the new approach can guide physicians as to which combination of drugs will most likely produce the best outcome.

The next step in the research is to validate the new method in samples from cancer patients who have been treated with one of the pathway-specific drugs to determine if the pathway predictors are able to select those patients most likely to respond to the drug. A positive result would then form the basis for a clinical study that would evaluate the effectiveness of the pathway prediction to guide the most effective use of therapeutics.

16.2.10 Role of Molecular Imaging in Personalized Therapy of Cancer

In oncology, if cancer cells are removed from their microenvironment, their pattern of gene expression changes because the behavior of tumor cells is inextricably linked to their environments. Therefore, noninvasive, quantitative means of detecting gene and protein activity are essential. In vivo imaging is one method for achieving this. Various technologies available for this purpose are PET scanning, single photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI). Ultrasound and CT are being reengineered to reflect information at the cellular level. In vivo optical imaging technologies have matured to the point where they are indispensable laboratory tools for small animal imaging. Human applications are being explored and the future for clinical optical imaging techniques looks bright. Merging these molecular imaging techniques with minimally or noninvasive image-guided therapeutic delivery techniques is an important goal in the fight against cancer.

In investigational and clinical oncology, there is a need for imaging technologies that will indicate response to therapy prior to clinical evidence of response. The conventional imaging methods such as CT and MRI enable anatomic measurements of the tumor. This may be useful for assessing response to traditional cytotoxic agents where tumor shrinkage occurs early. In contrast to this, molecularly targeted agents tend to induce arrest of cancer cell growth and development, but not necessarily significant tumor shrinkage in the short term. Thus there is a need for functional or molecular imaging methods that would give information about what is happening in the tumor at the molecular level. One example of this approach is an attempt to find an explanation for poor performance of some antiangiogenesis drugs in clinical trials despite abundant preclinical evidence that the drugs should work. Noninvasive molecular imaging is needed to identify patients that are suitable for a particular targeted therapy and to determine if the drug is reaching its target and in sufficient quantities to block the target. The molecularly targeted approaches enable the therapy to be individually tailored to a given patient's tumor and metabolism.

16.2.10.1 Functional Diffusion MRI

Functional diffusion MRI scan (Molecular Imaging Products) could help physicians decide quickly whether treatment for brain tumors is having any effect. The scan uses MRI to track the Brownian motion of water through the brain. Tumor cells block the flow of water, so as those cells die, water diffusion patterns change, and the new MRI technology can track it. Application of this technique in patients with malignant brain tumors showed changes in the diffusion map if chemotherapy or radiation therapy was having any effect. It worked within 3 weeks, 10 weeks before traditional MRI techniques of assessing whether therapy is working. Usually, patients get 7 weeks of treatment, followed by a traditional MRI scan 6 weeks afterward to see if the tumor has shrunk. If it does not, the management approach may be altered depending on the tumor. Speeding up this process can save patients from often-uncomfortable treatments that may be a waste of time. The use of MRI tumor diffusion values to accurately predict the treatment response early on would enable some patients to switch to a more beneficial therapy and avoid the side effects of a prolonged and ineffective treatment.

16.2.10.2 FDG-PET/CT for Personalizing Cancer Treatment

Multimodality imaging, as represented by PET, has a definite role in the evaluation of a patient with cancer. Fluorodeoxyglucose (FDG)-PET is rapidly becoming the key investigative tool for the staging and assessment of cancer recurrence. In the last 5 years, PET has also gained widespread acceptance as a key tool used to demonstrate early response to intervention and therapy, whereas changes in size of tumor as shown by CT alone may take longer. This clinical need is being addressed with FDG-PET/CT because of its inherent ability to demonstrate (before other biomarkers of response) if disease modification has occurred (Ben-Haim and Ell 2009). This is an important factor in personalizing cancer treatment.

In NSCLC, reduction of metabolic activity as demonstrated by FDG-PET after one cycle of chemotherapy is closely correlated with final outcome of therapy. Using metabolic response as an endpoint may shorten the duration of phase II studies evaluating new cytotoxic drugs and may decrease the morbidity and costs of therapy in nonresponding patients. Another example of a generic functional imaging method is the use of FDG-PET to look at the response of gastrointestinal stromal tumor (GIST) to Gleevec. Preliminary studies show marked decrease of FDG uptake in GIST tumors within 24 h in patients who go on to show clinical response to Gleevec. PET can accurately diagnose tumor response in 85 % of patients. Radiolabeled annexin V may provide an early indication of the success or failure of anticancer therapy on a patient-by-patient basis as an in vivo marker of tumor cell killing. The temporal patterns of tumor cell loss has been demonstrated by SPECT and provides a better understanding of the timing of radiolabeled annexin V uptake for its development as a marker of therapeutic efficacy.

Abnormal tryptophan metabolism catalyzed by indoleamine 2,3-dioxygenase may play a prominent role in tumor immunoresistance in many tumor types, including lung tumors. Prolonged retention of alpha-(11)C-methyl-l-tryptophan (AMT), a PET tracer for tryptophan metabolism, in NSCLCs suggests high metabolic rates of tryptophan in these tumors. AMT PET/CT may be a clinically useful molecular imaging method for personalized cancer treatment by identifying and monitoring patients who have increased tumor tryptophan metabolism and are potentially sensitive to immunopharmacotherapy with indoleamine 2,3-dioxygenase inhibitors (Juhász et al. 2009).

16.2.10.3 Image-Guided Personalized Drug Delivery in Cancer

Image-guided drug delivery (IGDD) in cancer is a form of individualized therapy where imaging methods are used in guidance and monitoring of localized and targeted delivery of therapeutics to the tumor. A systematic approach to IGDD requires mechanisms for targeting, delivery, activation, and monitoring of the process. Although the goal in IGDD is to optimize the therapeutic ratio through personalized image-guided treatments, a major challenge is in overcoming the biological barriers to the delivery of therapeutics into tumors and cells. Physiologic and quantitative imaging techniques may serve as enabling tools that could potentially transform many existing challenges into opportunities for advancement of the field (Tandon and Farahani 2011).

16.2.10.4 Tumor Imaging and Elimination by Targeted Gallium Corrole

Sulfonated gallium(III) corroles are intensely fluorescent macrocyclic compounds that spontaneously assemble with carrier proteins to undergo cell entry. In vivo imaging and therapeutic efficacy of a tumor-targeted corrole noncovalently assembled with a heregulin-modified protein directed at the human EGFR. Systemic

delivery of this protein–corrole complex results in tumor accumulation, which can be visualized in vivo owing to intensely red corrole fluorescence. Targeted delivery in vivo leads to tumor cell death while normal tissue is spared in contrast with the effects of doxorubicin, which can elicit cardiac damage during therapy and required direct intratumoral injection to yield similar levels of tumor shrinkage compared with the systemically delivered corrole (Agadjanian et al. 2009). The targeted complex ablated tumors at >5 times a lower dose than untargeted systemic doxorubicin, and the corrole does not damage heart tissue. Complexes remain intact in serum and the carrier protein elicits no detectable immunogenicity. The sulfonated gallium(III) corrole functions for both tumor detection and intervention with safety and targeting advantages over standard chemotherapy.

16.2.10.5 Future Prospects of Molecular Imaging in Management of Cancer

Molecular imaging can improve therapeutic strategies that provide better patient selection for therapeutic personalization than conventional methods and provides a variety of new tools to accelerate the development of cancer therapies. The recent drive to develop molecular imaging probes and standardize molecular imaging techniques is creating the scaffolding for the evolving paradigm shift to personalized cancer therapy (Kurdziel et al. 2008). Molecular imaging, also referred to by the term "virtual biopsy," can provide a more complete systematic picture of the living tumor, but it is not likely to replace and would rather be complementary to pathology, IHC, and genomic analysis. However, molecular imaging methods are certainly complementary to biopsy. The primary advantages of molecular imaging are that it is nondestructive, noninvasive, or minimally invasive, and thus easier on patients; permits the collection of data over time, thus enabling post-therapy evaluations; provides near real-time functional information; and encompasses large volumes of tissue (the whole body in most cases). One drawback of the molecularly targeted approaches is the expensive development and lack of interest in the pharmaceutical industry to combine functional imaging with anticancer drugs in development.

16.2.11 Unraveling the Genetic Code of Cancer

A systematic analysis was carried out for determining the sequence of well-annotated human protein-coding genes in two common tumor types to identify genetic alterations in breast cancer and CRC (Sjoblom et al. 2006). Analysis of 13,023 genes in 11 breast cancer and 11 CRC revealed that individual tumors accumulate an average of approximately 90 mutant genes but that only a subset of these contribute to the neoplastic process. Using stringent criteria to delineate this subset, the authors identified 189 genes (average of 11 per tumor) that were mutated at significant frequency. The vast majority of these genes were not known to be

genetically altered in tumors and are predicted to affect a wide range of cellular functions, including transcription, adhesion, and invasion. These data define the genetic landscape of two human cancer types, provide new targets for diagnostic and therapeutic intervention, and open fertile avenues for basic research in tumor biology. The mutated genes in breast and colon cancers were almost completely distinct, suggesting very different pathways for the development of each of these cancer types. Each individual tumor appeared to have a different genetic blueprint, which could explain why cancers can behave very differently from person to person. The discovery could also lead to better ways to diagnose cancer in its early, most treatable stages and personalized treatments. Maximizing the numbers of targets available for drug development in a specific cancer means that patients will ultimately receive more personalized, less toxic drugs.

16.3 Cancer Prognosis

Molecular diagnostics provide an easier, less invasive way to determine cancer prognosis. For example, patients with the greatest degree of amplification (in terms of gene copy numbers) of the N-*myc* gene in neuroblastoma, a highly malignant tumor, have the worst prognosis. Molecular tests for TP53 and RER are already considered to offer prognostic value in certain types of cancer. In addition, the ability to locate residual cancer by molecular methods can aid in predicting the course of the disease.

A more accurate means of prognosis in breast cancer will improve the selection of patients for adjuvant systemic therapy. Using microarray analysis to evaluate a previously established 70-gene prognosis profile, a series of consecutive patients with primary breast carcinomas have been classified as having a gene expression signature associated with either a poor prognosis or a good prognosis. The gene expression profile (tumor signature) was found to be a more powerful predictor of the outcome of disease in young patients with breast cancer than standard systems based on clinical and histological criteria. Currently, 70–80 % of patients that receive adjuvant therapy would have survived without it, and chemotherapy has significant side effects and long-term consequences. This classification method can predict those that should receive treatment as effectively as other methods, while reducing the number who receive treatment unnecessarily. Gene signatures therefore seem to be the way forward in predicting outcome and should pave the way for new therapies that are tailored to the patient.

Gene expression profiles based on microarray analysis can be used to predict patient survival in early-stage lung adenocarcinomas (LADs). Genes most related to survival are identified with univariate Cox analysis. The identification of a set of genes that predict survival in early-stage LAD enables the delineation of a high-risk group that may benefit from adjuvant therapy. Differentially expressed genes were used to generate a 186-gene “invasiveness” gene signature (IGS), which is strongly associated with metastasis-free survival and overall survival for four different types

of tumors: breast cancer, medulloblastoma, lung cancer, and prostate cancer (Liu et al. 2007). The prognostic power of the IGS was increased when combined with the wound-response signature based on transcriptional response of normal fibroblasts to reveal links between wound healing and cancer progression.

16.3.1 Detection of Mutations for Risk Assessment and Prevention

Tests with the greatest potential for risk assessment include those that target mutations in the following genes:

- BRCA1 and BRCA2 (for breast and ovarian cancers)
- MLH1 and MSH2 (for colon cancer)
- APC (for familial adenomatous polyposis)
- RET (for medullary thyroid cancer)
- TP53 (for several tumors)
- CDKN2A (for melanoma)
- RB1 (for retinoblastoma)

Detection of mutation in an individual would theoretically lead to increased surveillance. Lifestyle changes might be advised to avoid known risk factors for progression of cancer. In some cases, prophylactic surgery may be recommended. In addition, some chemotherapeutic agents might be prescribed on a preventive basis. Detection of a mutation may be followed by surveillance-oriented examinations, including those involving colonoscopy, mammography, measurement of PSA, and other tests. This tactic will promote the early detection of cancer and early management. Current molecular research is expected to reveal other markers for early diagnosis of cancer. In addition, the possibility of generating genetic profiles for individual tumors offers unique opportunities for distinguishing between metastases and primary tumors.

Effective targeted cancer therapeutic development depends upon distinguishing disease-associated "driver" mutations, which have causative roles in pathogenesis of malignancy, from "passenger" mutations, which are dispensable for cancer initiation and maintenance. Translational studies of clinically active targeted therapeutics can definitively discriminate driver from passenger lesions and provide valuable insights into human cancer biology.

16.4 Impact of Biomarkers on Management of Cancer

Biomarkers are playing an important role in the diagnosis as well as management of cancer. Biomarkers based on integration of genomic and proteomic alterations are useful for determining tumor prognosis and prediction of response to therapy. Some examples of applications of biomarkers in the personalized management of cancer are given here.

16.4.1 HER-2/neu Oncogene as a Biomarker for Cancer

HER-2/neu oncogene, also referred to as c-erbB-2, encodes a protein with a molecular weight of 185,000 Da and is structurally related to the human epithelial growth factor receptor. The full-length p185 HER-2/neu protein is composed of a cytoplasmic domain with tyrosine kinase activity, a transmembrane domain, and an extracellular domain (ECD) that is shed from the surface of breast cancer cells. Numerous studies have shown that the shed ECD of HER-2/neu is a glycoprotein with a molecular weight between 97 and 115 kDa and designated p105. The ECD can be accurately quantified in serum with an ELISA that uses monoclonal antibodies (MAbs) directed to the external epitopes of the HER-2/neu protein. Many publications show that the ECD is shed into the blood of normal individuals and can be elevated in women with metastatic breast cancer. Many of these serum HER-2/neu studies have confirmed the substantial data from tissue studies that HER-2/neu is a biomarker of poor prognosis, shorter overall survival, and biological aggressiveness. Scientific studies suggest that quantitation of the ECD may have several important clinical applications such as monitoring breast cancer patients with metastatic disease.

Various reports have shown that 30–50 % of women with positive HER-2/neu tumors at primary diagnosis develop elevated levels of serum HER-2/neu with progression to metastatic breast cancer. These studies have also illustrated that monitoring serum ECD levels post-surgery correlated with clinical course of disease and that serum HER-2/neu levels were observed to increase with disease progression or to decrease with response to therapy. Several reports also show that elevated levels of serum HER-2/neu can occur in women with metastatic breast cancer that had primary breast tumors that were negative for HER-2/neu expression by IHC. According to many IHC and serum studies, the HER-2/neu protein is overexpressed in many tumors of epithelial origin including lung, prostate, pancreatic, colon, stomach, ovarian, and hepatocellular cancer.

16.4.2 Cancer Treatment Guided by Asparagine Synthetase as a Biomarker

L-Asparaginase (L-ASP), a bacterial enzyme used to treat ALL, selectively starves cells that cannot synthesize sufficient asparagine for their own needs. Studies show that cancer cells that contain less asparagine synthetase (ASNS) are more susceptible to L-ASP. The response to L-ASP therapy is often better when the expression of ASNS is limited. However, there is conflicting data from patient samples with regard to correlation between ASNS mRNA content and ASNase sensitivity. A study has shown that it is important to measure ASNS protein rather than mRNA in predicting ASNase responsiveness (Su et al. 2008).

A new method has been described for enhancing L-ASP activity by combining it with antagonists of ASNS, such as small interfering RNAs (siRNAs), antisense

nucleotides, antibodies, or small-molecule inhibitors for the treatment of cancer (Lorenzi et al. 2006). Reducing or suppressing the expression of ASNS potentiates the growth inhibitory activity of L-ASP four- to fivefold. Tissue microarrays confirmed low ASNS expression in a subset of clinical ovarian cancers as well as other tumor types. Overall, this pharmacogenomic/pharmacoproteomic study suggests the use of L-ASP for personalized treatment of a subset of ovarian cancers (and perhaps other tumor types), with ASNS as a biomarker for the selection of patients most likely to respond to L-ASP treatment. The technology is currently in the preclinical stage of development. Phase I clinical trials were initiated using L-ASP in combination with gemcitabine for the treatment of patients with solid tumors.

16.4.3 Oncogene GOLPH3 as a Cancer Biomarker

Genome-wide copy number analyses of human cancers have identified frequent 5p13 amplification in several solid cancers, including lung, ovarian, breast, prostate, and melanoma. Using integrative analysis of a genomic profile of the region, scientists have identified a Golgi protein, GOLPH3, as a candidate targeted for amplification (Scott et al. 2009). Gain- and loss-of-function studies in vitro and in vivo validated GOLPH3 as a potent oncogene. Physically, GOLPH3 localizes to the trans-Golgi network and interacts with components of the retromer complex, which has been linked to target of rapamycin (TOR) signaling. GOLPH3 regulates cell size, enhances growth factor-induced mammalian target of rapamycin (mTOR) (also known as FRAP1) signaling in human cancer cells, and alters the response to an mTOR inhibitor in vivo. Thus, genomic and genetic, biological, functional, and biochemical data in yeast and humans establishes GOLPH3 as a new oncogene that is commonly targeted for amplification in human cancer and is capable of modulating the response to rapamycin, a cancer drug in clinical use. A protein made from GOLPH3 may serve as a biomarker for tumors that can be effectively treated with the rapamycin: tumors with a high level of the protein are more apt to shrink in response to the drug than those with low levels.

16.4.4 Predictive Biomarkers for Cancer

Unpredictable efficacy and toxicity are hallmarks of most anticancer therapies. Predictive markers are factors that are associated with response or resistance to a particular therapy. Currently, the only recommended predictive markers in oncology are estrogen receptor (ER) and progesterone receptor (PR) for selecting endocrine-sensitive breast cancers and HER-2 for identifying breast cancer patients with metastatic disease who may benefit from trastuzumab (TCH). For malignancies other than breast cancers, validated predictive markers are not available as yet.

16.4.5 Sequencing for Discovering Biomarkers to Personalize Cancer Treatment

A team of researchers from Johns Hopkins University has developed a sequencing-based method for personalized analysis of rearranged ends (PARE) in individual tumors, identifying biomarkers that could subsequently be used to track cancer using patient blood samples. The approach relies on massively parallel sequencing by the use of SOLiD platform (Applied Biosystems/Life Technologies) to find translocations and rearrangements in solid tumors. After finding such rearrangements in breast cancer and CRC samples, PARE is used to track cancer treatment response, recurrence, and metastasis in CRC patients. This is an important step in bringing new genome sequencing technologies to personalized patient care. Most tumors do not contain rearrangements, but when they occur, the location of these rearrangements varies from one individual to the other, making them good biomarker candidates. Previous studies on sequencing individuals' genomes were focused on single-letter changes, but this study looked for the swapping of entire sections of the tumor genome. After mapping about 40 million mate-pair sequence reads per sample to version 18 of the human reference genome, the team sifted through the sequence looking for signs of rearrangements, such as copy number changes or situations in which mate-pair tags mapped to different parts of the genome. In the process, they found an average of 14 rearrangements per tumor. Of these, roughly half were intra-chromosomal rearrangements and half were inter-chromosomal. When the researchers expanded their analysis to include two more colorectal tumor samples, they found an average of nine rearrangements per sample or between four and 15 rearrangements in each tumor.

The researchers were also able to find rearrangements in two of the CRC genomes even without sequencing matched normal samples. They then designed primers corresponding to rearrangement breakpoints in the tumor sequence and investigated whether they could detect these sequences when tumor DNA was diluted by normal DNA as it would be in blood or other bodily fluids. They could detect one cancer genome equivalent even when dwarfed by some 390,000 normal copies of the genome. They also showed that they could use the same PCR-based approach to detect rearrangements in actual blood samples from two of the CRC patients, e.g., by testing blood samples from one CRC patient before and after surgery, the team was able to follow the individual's treatment. Before surgery, DNA from the patient's blood sample contained a chromosome 4–chromosome 8 fusion corresponding to the individual's cancer. Immediately after surgery, the levels of the mutant DNA fusion dropped off but increased a few days later as the cancer recurred. When the individual underwent chemotherapy, this treatment again curbed the levels of the fusion biomarker, though a small amount remained due to metastatic lesions in the individual's liver. Because most clinically important tumors are thought to contain DNA rearrangements, the PARE approach holds promise for finding patient-specific biomarkers that can be used to improve the treatment of a variety of cancers.

The cost of PARE was reportedly around $5,000 per assay, though the cost is expected to go down as sequencing prices drop and read quality and length improve. As PARE becomes affordable, it will be a helpful addition for physicians to tailor patient care and may become a useful supplement to traditional monitoring by imaging or other approaches. The method holds potential for monitoring cancer and guiding treatment, e.g., it may help differentiate between individuals whose cancers are cured by surgery alone and those who require follow-up with aggressive chemotherapy or radiation following surgery. PARE will become available for many cancer patients in the near future, depending largely on sequencing costs.

16.4.6 VeraTag™ Assay System for Cancer Biomarkers

The VeraTag™ assay system (Monogram Biosciences) is a high-performance, high-throughput system for studies of gene expression and protein expression and for applications such as cell signaling and pathway activation, protein–protein interaction, and cell receptor binding. The system uses proprietary VeraTag™ reporters to multiplex the analysis of genes and/or proteins from the same sample. The VeraTag™ assay system is ideally suited to analysis of complex biology such as that seen in cancer. These unique assays can precisely measure many types of pathway biomarkers simultaneously—using small samples, such as those obtained from standard tumor biopsies. These biomarkers could be used to correlate disease type and progression, resulting in improved treatment. Novel VeraTag™ assays for unique protein biomarkers such as receptor complexes and phosphorylation events are being developed to focus on profiling EGFR family signal transduction pathways. Further research will be aimed at applying VeraTag™ assays to retrospective analysis of patient samples for validation and diagnostic development. It can accelerate the development of targeted therapeutics, improve clinical trial design and results, clarify and individualize the selection of medications, and optimize outcomes for patients with cancer.

16.5 Determination of Response to Therapy

Several approaches have been investigated for predicting and monitoring response to anticancer chemotherapy. Some of these are described here.

16.5.1 Biomarker-Based Assays for Predicting Response to Anticancer Therapeutics

The high incidence of resistance to DNA-damaging chemotherapeutic drugs and severe side effects of chemotherapy have led to a search for biomarkers able to predict which patients are most likely to respond to therapy.

ERCC1–XPF nuclease is required for nucleotide excision repair of DNA damage by cisplatin and related drugs, which are widely used in the treatment of cancer. The levels of ERCC1–XPF in a tumor could indicate whether it will be sensitive or resistant to a certain chemotherapeutic agent. Although several commercially available antibodies are suitable for immunodetection of ERCC1–XPF in some applications, only a select subset is appropriate for detection of this repair complex in fixed specimens. A study provides reliable tools for clinicians to measure the enzyme ERCC1–XPF as a biomarker in clinical specimens that could help stratify patients according to cancer risk, response to treatment, and overall prognosis (Bhagwat et al. 2009).

Capecitabine (Roche's Xeloda) is a novel, oral fluoropyrimidine carbamate rationally designed to generate 5-fluorouracil (5-FU) preferentially in tumor tissue via a three-step enzymatic cascade. Roche is investigating diagnostic tests based on various biomarkers—thymidylate synthase (TS), thymidine phosphorylase (TP), and dihydropyrimidine dehydrogenase (DPD)—to predict responders to this therapy. Proof-of-principle studies for the biomarkers are running concomitantly with clinical trials of capecitabine.

16.5.2 ChemoFx Cell Culture Assay for Predicting Anticancer Drug Response

ChemoFx assay (Precision Therapeutics Inc. (PTI)) is a phenotype-based cell culture assay for predicting anticancer drug responses in individual cancer patients. The ChemoFx assay is based on the outgrowth and short-term primary culture of epithelial cells derived from pieces of solid tumors that are obtained at the time of tumor resection. The tumor cells are isolated and maintained in short-term culture before drug testing and their epithelial identity is verified by immunohistochemical staining methods such as the presence of cytokeratin, an intermediate filament protein of epithelial cells, for carcinomas.

Malignant epithelial cells are grown attached in wells of microtiter plates and treated with six escalating doses of chemotherapeutic drug. Using an operator-controlled automated image analysis system, cell kill is measured microscopically by counting the number of live cells remaining after dead cells have detached and are subsequently rinsed away. A dose–response graph is automatically generated by comparing the number of cells in drug-treated wells with those in control wells.

16.5.3 Ex Vivo Testing of Tumor Biopsy for Chemotherapy Sensitivity

Many oncologists are beginning to believe that new techniques to evaluate tumors' responses to chemotherapeutic agents promise a future of personalized cancer management. Rational Therapeutics' EVA® assay measures apoptotic events that occur

as a result of drug exposure. Hence, highly responsive cancers are those with the greatest degree of apoptosis in the laboratory. The company has developed a novel regimen for refractory ovarian cancers—gemcitabine plus cisplatin. Study results showed a correlation between ex vivo sensitivity and resistance and patient outcome. The Gynecologic Oncology Group, a multicenter nonprofit organization sponsored by the National Cancer Institute, is conducting a national clinical trial of the gemcitabine plus cisplatin combination for the treatment of relapsed ovarian cancer. The idea of the assays in predicting chemosensitivity continues to grow. It has not been used for first-line treatment for ovarian cancer yet because it has not been proven that anything is more effective than platinum and Taxol. But assays can provide valuable information for its selection as a second-line treatment. The lack of efficacy of the drug could be due to the drugs' inability to be delivered to the tumor or inappropriate levels of drug. In 50–60 % of the instances, a drug is not effective in vivo even though the in vitro assays predict efficacy.

ChemoFx assay (Precision Therapeutics) is an ex vivo assay designed to predict the sensitivity and resistance of a given patient's solid tumor to a variety of chemotherapy agents (Brower et al. 2008). A portion of a patient's solid tumor, as small as a core biopsy, is mechanically disaggregated and established in primary culture where malignant epithelial cells migrate out of tumor explants to form a monolayer. Cultures are verified as epithelial and exposed to increasing doses of selected chemotherapeutic agents. The number of live cells remaining posttreatment is enumerated microscopically using automated cell-counting software. The resultant cell counts in treated wells are compared with those in untreated control wells to generate a dose–response curve for each chemotherapeutic agent tested on a given patient specimen. Features of each dose–response curve are used to score a tumor's response to each ex vivo treatment as "responsive," "intermediate response," or "nonresponsive." Collectively, these scores are used to assist an oncologist in making treatment decisions.

16.5.4 Genomic Approaches to Predict Response to Anticancer Agents

16.5.4.1 Gene Expression Patterns to Predict Response of Cancer to Therapy

Human lymphoblastoid cells, immortalized white blood cell lines derived from different healthy individuals, display considerable variation in their transcription profiles, which underlies interindividual susceptibility to DNA-damaging agents. Gene expression, measured by Affymetrix GeneChip Human Genome U133 Plus 2.0, has been associated with sensitivity and resistance to DNA-damaging anticancer agents (Fry et al. 2008). A cell line from one person would be killed dramatically, while that from another person can be resistant to exposure to the anticancer agent. Using computational models to pinpoint differentially expressed genes with positive or negative correlations, the investigators identified 48 genes whose pretreatment

expression could predict sensitivity to anticancer agent MNNG with 94 % accuracy. MNNG alkylates certain DNA bases, leading to mutagenesis. Some of this damage can be repaired by the DNA methyltransferase Methyl Guanine Methyl Transferase (MGMT). But if it is not, the DNA mismatch repair or MMR pathway targets damaged DNA bases and sets off apoptosis. Consequently, cells with reduced MGMT activity but a functional MMR pathway are expected to be more sensitive to MNNG, whereas cells deficient in both pathways are more MNNG resistant but accumulate mutations when exposed to the compound. Because gene expression is the most accurate predictor of alkylation sensitivity, there are good prospects for translating these findings to a clinical setting to predict whether a tumor will respond to alkylation chemotherapy.

16.5.4.2 Genomic Analysis of Tumor Biopsies

Genomic Health Inc. is developing a service to provide individualized genomic analysis of tumor biopsies to physicians as a guide to the treatment of patients with cancer. Fixed paraffin-embedded tissues (FPET), stored tumor tissue samples collected over the past 20 years, are used for this purpose. Instead of waiting years to accumulate fresh tissue and track patient outcomes, Genomic Health's FPET analysis can be performed using routinely stored biopsies from patients with known outcomes therefore accelerating clinical trials. RNA analysis of thin sections of standard tumor biopsies is used to evaluate panels of genes that may predict breast cancer recurrence and response to chemotherapy as well as response to EGFR inhibitor therapy in lung cancer. This approach is now being tested in clinical trials on patients with breast cancer and lung cancer. This technology will allow physicians to tailor the treatment and prognosis for an individual patient, using a small panel of genes selected from thousands of genes.

Activating mutations of KIT or kinase platelet-derived growth factor receptor alpha (PDGFRA) are found in the vast majority of GISTs, and the mutational status of these oncoproteins is predictive of clinical response to imatinib. PDGFRA mutations can explain response and sensitivity to imatinib in some GISTs lacking KIT mutations.

16.5.4.3 Genotype-Dependent Efficacy of Pathway Inhibition in Cancer

Therapeutic inhibition of genetically activated oncoproteins can induce massive apoptosis of tumor cells, which may lead to dramatic regression of cancer. The phosphatidylinositol 3-kinase (PI3K) and MAPK signaling pathways are central regulators of oncogenic transformation and tumor maintenance. Systematic linking of drug response to genomic aberrations in NSCLC, as well as in cell lines of other tumor types and in a series of in vivo cancer models, has shown that tumors with genetically activated receptor tyrosine kinase (RTKs) depend on PI3K signaling, whereas tumors with mutations in the RAS/RAF axis depend on MAPK signaling (Sos et al. 2009). However, the efficacy of downstream pathway inhibition is limited

by the release of negative feedback loops on the reciprocal pathway. By contrast, combined blockade of both pathways can overcome the reciprocal pathway activation induced by inhibitor-mediated release of negative feedback loops and results in a significant increase in tumor apoptosis. Thus, by using a systematic chemogenomic approach, genetic lesions connected to PI3K and MAPK pathway activation can be identified and provide a rationale for combined inhibition of both pathways. These findings may have implications for patient stratification in clinical trials.

16.5.4.4 Mutation Detection at the Molecular Level

It is known that genetic mutations are responsible for sensitizing some tumor cells to chemotherapy, while other mutations render tumor cells completely resistant to drug treatments. Research progress in this area has been slow because analysis of DNA from tumors is complicated by varying amounts of tumor cells in patient samples. Furthermore, the heterogeneous nature of many tumors makes it difficult to accurately sequence the tumor DNA, which is required in order to personalize treatment. This is compounded by cost-prohibitive, conventional low-resolution sequencing methods that lack sufficient accuracy to characterize the DNA in cancerous cells. Next-generation sequencing (NGS) can be used for the detection of cancer gene mutations present at extremely low levels. Microreactor-based 454 Sequencing™ (Roche) advanced sequencing technology can generate hundreds of thousands of DNA sequences in one run, rapidly and comprehensively conducting high-throughput nucleotide sequencing, with specific application to sequencing of whole genomes and ultradeep sequencing of target genes. The method is not only very sensitive, but it is also quantitative and provides a digital display of gene variation within tumors. It identifies rare cancer-associated genetic variations at the molecular level, potentially enabling the personalization of targeted therapies. This technology has been used to analyze mutations in five exons of the EGFR gene in tumor samples from patients with lung cancer. The EGFR gene is the target for several new anticancer drugs called EGFR inhibitors. Thus 454 Sequencing may help to validate the ability of EGFR mutations to predict patient responsiveness to treatment with an EGFR inhibitor. Ultimately, this system will enable personalized medicine, such as identifying the early stages of drug resistance and facilitating a change in treatment that is tailored to a patient's unique genetic response.

16.5.4.5 RNA Disruption Assay™

Rna Diagnostics Inc. has developed RNA Disruption Assay™ (RDA™) that enables determination of efficacy of chemotherapy within the first three cycles and helps in making decision about further continuation of therapy. The test is based on the observation that in some patients chemotherapy administration results in marked degradation of tumor RNA, indicating a positive response and tumor destruction.

16.5.4.6 Role of Genetic Variations in Susceptibility to Anticancer Drugs

Genetic variations in susceptibility to anticancer drugs have been investigated using a genome-wide model of human lymphoblastoid cell lines from the International HapMap consortium, of which extensive genotypic information is available (Huang et al. 2007). This model integrated genotype, gene expression, and sensitivity of HapMap cell lines to drugs. Associations were evaluated between genotype and cytotoxicity, genotype and gene expression, and gene expression of the identified candidates was correlated with cytotoxicity. The analysis identified 63 genetic variants that contribute to etoposide-induced toxicity through their effect on gene expression. These include genes that may play a role in cancer (AGPAT2, IL1B, and WNT5B) and genes not yet known to be associated with sensitivity to etoposide. This method can be used to elucidate genetic variants contributing to a wide range of cellular phenotypes induced by chemotherapeutic agents.

16.5.5 Nongenetic Factors for Variations in Response of Cancer Cells to Drugs

It is well known that not all cells of a particular cell type react to cancer treatments uniformly and genetics alone cannot explain sensitivity or resistance to chemotherapy. In the case of apoptosis mediated by TRAIL (tumor necrosis factor (TNF)-related apoptosis-inducing ligand), it is common for some cells in a clonal population to die while others survive—a striking divergence in cell fate. Among cells that die, the time between TRAIL exposure and caspase activation is highly variable. Imaging of sister cells expressing reporters of caspase activation and mitochondrial outer membrane permeabilization after exposure to TRAIL has shown that naturally occurring differences in the levels or states of proteins regulating receptor-mediated apoptosis are the primary causes of cell-to-cell variability in the timing and probability of death in human cell lines (Spencer et al. 2009). Protein state is transmitted from mother to daughter, giving rise to transient heritability in fate, but protein synthesis promotes rapid divergence so that sister cells soon become no more similar to each other than pairs of cells chosen at random. These results have implications for understanding “fractional killing” of tumor cells after exposure to chemotherapy and indicate that the genetic identity of a tumor cell is an incomplete predictor for how it will respond to certain treatments. These findings also offer an alternative to the cancer stem cell (CSC) hypothesis, which states that certain cancers survive standard treatments because a population of tumor-specific stem cells evades chemotherapy or radiation. This study, however, offers an alternative explanation, that certain cells produce quantities of proteins purely through chance, which fundamentally alter the cell’s response to treatment. This new insight will make it possible to design anticancer treatments that are more effective than those currently available.

16.5.6 Proteomic Analysis of Tumor Biopsies to Predict Response to Treatment

Protein analysis of malignant tissue and the discovery of protein signatures have been used for assessing the stage of disease as well as their correlation with patient survival. Protein profiles have been obtained from human gliomas of various grades through direct analysis of tissue samples using matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). Statistical algorithms applied to the MS profiles from tissue sections identified protein patterns that correlated with tumor histology and patient survival. Two patient populations, a short-term and a long-term survival group, were identified based on the tissue protein profiles. In addition, a subset of patients diagnosed with high-grade, grade IV, malignant gliomas were analyzed, and a novel classification scheme that segregated short-term and long-term survival patients based on the proteomic profiles was developed. The protein patterns described served as an independent indicator of patient survival. These results show that this new molecular approach to monitoring gliomas can provide clinically relevant information on tumor malignancy and is suitable for high-throughput clinical screening.

16.5.7 Real-Time Apoptosis Monitoring

There is a need for a real-time monitoring of apoptosis because of the serious problems that result from not knowing if and when anticancer therapy starts to work. For the patient, receiving a therapy that is not effective means unnecessary suffering, from both the tumor continuing to grow and any side effects that accompany the ineffective treatment. Receiving ineffective therapy for longer than needed also delays the start of second-line therapies that might work. Worse still, the failed treatment can trigger genetic defense mechanisms in tumor cells that can make it resistant to second-line therapies using other drugs. This phenomenon is known as cross-resistance.

The current months-long lag between the start of therapy and the appearance of obvious signs of initial success or failure also affects how new therapies undergo clinical testing. Because of the possibility of cross-resistance, the Food & Drug Administration (FDA) is reluctant to allow testing of new cancer therapies on anyone but on those patients who have exhausted all other therapeutic possibilities. Unfortunately, such patients are far less likely to respond to any therapy, making it far more difficult to prove the benefits of an experimental therapy. This difficulty is particularly true for the new generation of molecularly targeted therapies that aim to stop tumor growth early in its progression. An available real-time apoptosis monitoring might enable such drugs to be tested at the initial diagnosis of cancer with less concern that prolonged therapy, should it fail to work, would put patients at risk by letting their cancers grow unchecked for longer than necessary. Instead, getting an early sign that such an early therapy is not working would allow patients to

receive conventional therapy more quickly. Recognizing such a need, the National Cancer Institute's (NCI) Unconventional Innovations Program is funding the development of an apoptosis detector.

16.5.8 Serum Nucleosomes as Indicators of Sensitivity to Chemotherapy

In the nucleus of eukaryotic cells, DNA is associated with several protein components and forms complexes known as nucleosomes. During cell death, particularly during apoptosis, endonucleases are activated that cleave the chromatin into multiple oligo- and mononucleosomes. Subsequently, these nucleosomes are packed into apoptotic bodies and are engulfed by macrophages or neighboring cells. In cases of high rates of cellular turnover and cell death, they also are released into the circulation and can be detected in serum or plasma by Cell Death Detection ELISAplus (CDDE) from Roche Diagnostics. As enhanced cell death occurs under various pathologic conditions, elevated amounts of circulating nucleosomes are not specific for any benign or malignant disorder. However, the course of change in the nucleosomal levels in circulation of patients with malignant tumors during chemotherapy or radiotherapy (RT) is associated with the clinical outcome and can be useful for the therapeutic monitoring and the prediction of the therapeutic efficacy.

In patients with inoperable small-cell lung cancer, the efficacy of chemotherapy can be predicted early in the course of therapy by baseline values of serum nucleosomes as independent parameters. Prediction of efficacy of chemotherapy in NSCLC requires the following:

- Staging
- Age
- Baseline value of 1 CYFRA 21-1
- Area under the curve (AUC) of the values of nucleosomes on days 1–8

In advanced stage NSCLC, the initial level of serum CYFRA appears to provide more prognostic information than clinical stage. Furthermore, a drop in CYFRA after one cycle of therapy adds prognostic information, so that this threshold appears to be an early measure of response to chemotherapy.

16.5.9 Targeted Microbubbles to Tumors for Monitoring Anticancer Therapy

New strategies to detect tumor angiogenesis and monitor response of tumor vasculature to therapy are needed. Peregrine Pharmaceuticals' Vascular Targeting Agent technology using contrast ultrasound imaging with microbubbles targeted to tumor endothelium offers a noninvasive method for monitoring and quantifying vascular effects of antitumor therapy. The microbubbles are tiny lipid or albumin shells filled

with an inert gas that have a well-established safety record as contrast agents for ultrasound imaging applications, and they are currently widely used in cardiovascular medicine. Targeted microbubbles conjugated to MAbs were used to image and quantify vascular effects of two different antitumor therapies in pancreatic tumor-bearing mice treated with anti-vascular endothelial growth factor (anti-VEGF) MAbs and/or gemcitabine (Korpanty et al. 2007). Video intensity from targeted microbubbles correlated with the level of expression of the target (CD105, VEGFR2, or the VEGF–VEGFR complex) and with microvessel density in tumors under antiangiogenic or cytotoxic therapy. It was concluded that targeted microbubbles represent a novel and attractive tool for noninvasive, vascular-targeted molecular imaging of tumor angiogenesis and for monitoring vascular effects specific to antitumor therapy in vivo. This information could allow oncologists to modify patient treatment regimens soon after starting therapy, so that nonresponders could be switched to other therapies that might be more effective for them. The clinical development of contrast agents is typically faster than for therapeutics, and clinical trials of this approach could be feasible within 12–18 months. The potential of the approach is enhanced by the fact that the targeted microbubbles are "read" using ultrasound technology, which is widely available in most physicians' offices and is minimally invasive, safe, and cost-effective. The personalized medicine made feasible by this approach has the potential to increase the efficacy of cancer regimens, reduce side effects from ineffective treatments, and improve the overall cost-effectiveness of cancer therapy.

16.5.10 PET Imaging for Determining Response to Chemotherapy

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) and cytosine arabinoside (cytarabine, ara-C) represent a class of nucleoside analogs used in cancer chemotherapy. Administered as prodrugs, dFdC and ara-C are transported across cell membranes and are converted to cytotoxic derivatives through consecutive phosphorylation steps catalyzed by endogenous nucleoside kinases. Deoxycytidine kinase (DCK) controls the rate-limiting step in the activation cascade of dFdC and ara-C. DCK activity varies significantly among individuals and across different tumor types and is a critical determinant of tumor responses to these prodrugs. Current assays to measure DCK expression and activity require biopsy samples and are prone to sampling errors. Noninvasive methods that can detect DCK activity in tumor lesions throughout the body could circumvent these limitations. An approach to detecting DCK activity in vivo has been demonstrated by using PET and ^{18}F-labeled [1-(2′-deoxy-2′-fluoroarabinofuranosyl) cytosine] (^{18}FFAC), a DCK substrate with an affinity similar to that of dFdC as a PET probe (Laing et al. 2009). In vitro, accumulation of ^{18}FFAC in murine and human leukemia cell lines is critically dependent on DCK activity and correlates with dFdC sensitivity. In mice, ^{18}FFAC accumulates selectively in DCK-positive versus DCK-negative tumors, and ^{18}FFAC microPET scans can predict responses to dFdC. The results suggest that ^{18}FFAC PET might be useful for guiding treatment decisions in certain cancers by enabling individualized chemotherapy.

16.5.10.1 PET Imaging with Tyrosine Kinase Inhibitors

Several small-molecule tyrosine kinase inhibitors (TKIs) have been developed and approved. Treatment efficacies with TKI therapeutics are still too low and improvements require a personalized medicine approach. PET with radiolabeled TKIs (TKI-PET) is a tracking, quantification, and imaging method, which provides a unique understanding of the behavior of these drugs in vivo and of the interaction with their target(s). An overview of tracer synthesis and development indicated that each TKI requires a tailor-made approach (Slobbe et al. 2012). Moreover, current preclinical work and the first proof-of-principle clinical studies on the application of TKI-PET illustrate the potential of this approach for improving therapy efficacy and personalized cancer treatment.

16.5.11 Tissue Systems Biology Approach to Personalized Management of Cancer

Cellular Systems Biology (CSB™) applied to tissues has been named "Tissue Systems Biology" (TSB™) and involves the use of panels of fluorescence-based biomarkers that report the systems readout of patient samples. Cellumen Inc. (parent company of Cernostics) has successfully applied CSB™ to drug discovery, drug development, and personalized medicine over 3 years. Cernostics Pathology was creating a complete digital imaging pathology platform by integrating the best available components, while building advanced informatics tools to manage, mine, and classify patient tissue samples. The first diagnostic/therapeutic test being developed by Cernostics is a breast cancer test as part of collaboration with the Mayo Clinic.

16.6 Molecular Diagnostics Combined with Cancer Therapeutics

Basics of combination of diagnostics with therapeutics are discussed in Chap. 2. Cancer is a good example of such a combination, which would be useful for personalized management of cancer. Examples of technologies that can be used to combine diagnosis and therapeutics for cancer are listed below and will be discussed further under personalized management of various cancers:

- AmpliChip P53 as companion diagnostic for the anticancer drug Nutlin
- Flow cytometry testing for minimal residual disease (MRD) in chronic lymphocytic leukemia (CLL) treated with Campath
- Abl mutations testing in chronic myeloid leukemia (CML) for Gleevec resistance
- EGFR mutations testing in NSCLC for treatment with Tarceva/Iressa
- 5q fluorescent in situ hybridization (FISH) testing in myelodysplastic syndrome for lenalidomide (Revlimid) therapy

16.6.1 AmpliChip P53 as Companion Diagnostic for Cancer

Diagnosis of mutations in cancer genes is important for the development of companion diagnostics for cancer therapies. There are 400–500 genes mutated in cancer, which are mostly tumor suppressors. The best known tumor suppressor gene, p53, regulates ~150 other genes. P53 is mutated or deleted in approximately half of all cancers. Tumor cells with mutant p53 are typically unable to invoke apoptosis in response to DNA damage, rendering such tumors resistant to traditional chemotherapy and radiation therapy.

Advexin (Introgen Therapeutics) is a gene-based drug, injected directly into tumors, which uses an adenoviral vector to deliver the wild-type p53 gene to tumor cells. Patients with advanced squamous cell carcinoma of the head and neck cancer, whose pretreatment tumor samples overexpressed p53, were significantly more likely to respond to Advexin therapy than those whose tumor showed little p53 protein. The FDA has agreed to the use of Introgen's p53 molecular biomarkers in the analysis of Advexin clinical data used in support of submissions for approval. In updated data from phase II clinical trials, the predictive abnormal p53 biomarker was associated with a statistically significant increase in tumor responses to Advexin therapy. A reduction in tumor size was observed in 40 % of patients with the abnormal p53 biomarker compared to none (0 %) of the patients with p53 normal tumors (Nemunaitis et al. 2009). This makes p53 the first predictive biomarker for a gene-based drug. Not only is this a way to predict if the gene therapy is likely to succeed, the patients for which it does work are the most difficult ones to treat. Accumulation of p53 corresponds with a poor response to traditional therapies such as radiation and chemotherapy, as well as lower survival and a shorter time to disease progression. Advexin has also achieved 100 % response when combined with chemotherapy to treat locally advanced breast cancer and a 69 % response when used with radiation to treat NSCLC.

Roche is developing AmpliChip P53 as companion diagnostic for the anticancer drug Nutlin (RG7112 inhibitor), which regulates p53 by binding to MDM2.

16.6.2 Aptamers for Combined Diagnosis and Therapeutics of Cancer

Aptamers (derived from the Latin word "aptus" = fitting) are short DNA or RNA oligomers, which can bind to a given ligand with high affinity and specificity due to their particular three-dimensional structure and thereby antagonize the biological function of the ligand. Aptamers are beginning to emerge as a class of molecules that rival antibodies in both therapeutic and diagnostic applications. Aptamers are different from antibodies, yet they mimic properties of antibodies in a variety of diagnostic formats.

Researchers at the Open University (Milton Keynes, UK) have developed high-affinity aptamers as targeted therapeutics for the diagnosis, imaging, staging, and treatment of cancers including breast, bladder, and stomach cancers, as well as a

generic application for the treatment of abnormal growths with extensive potential future development. This method offers, apart from an immediate application in the diagnosis, imaging, and treatment of breast and other epithelial cancers, a generic application for the treatment of neoplastic disorders and extensive potential for future development. Combinatorial libraries have been used for the selection of aptamers that bind to a well-characterized and well-established cancer marker selectively and with high affinity. As part of their design, the aptamers are conjugated to ligands, molecules bearing binding sites for metal ions, to impart the therapeutic and diagnostic properties. In particular, stable chelation of technetium, rhenium, and yttrium radioisotopes results in novel radiopharmaceutical agents for imaging and selective cell kill as part of cancer diagnosis, imaging, and therapy. The use of paramagnetic gadolinium produces a novel, targeted MRI contrast agent that can achieve high local concentrations around the tumor site, thus offering high-definition imaging at lower gadolinium concentrations. The use of europium or terbium confers fluorescent properties to the aptamer complex, for use in diagnostic assays. These molecules offer significant advantages over existing antibody- and peptide-based recognition procedures in that they possess higher binding affinities to the target, leading to longer retention times and the ability to deliver a higher payload of the metal ion precisely to the target with a lower overall dose of the agent. The size of these molecules leads to reduced immunogenicity and increased tumor penetration, further enhancing their efficacy while minimizing potential side effects.

16.6.3 Monoclonal Antibodies for Combining Diagnosis with Therapy of Cancer

MAbs can be used for both diagnosing and targeting cancer and are described in Chap. 8. Two tests—Poteligeo Test IHC (immunohistochemistry) and Poteligeo Test FCM (Kyowa Medex)—were approved in Japan in 2012 as companion diagnostics for mogamulizumab (Kyowa Hakko Kirin's Poteligeo) injection, a therapeutic MAb for the treatment of adult T-cell leukemia (ATL). Poteligeo binds to CCR4, which is expressed on the surface of ATL cells, which are killed by MAb-dependent cell-mediated cytotoxicity. The companion diagnostic tests detect the presence of CCR4 expressed by ATL cells before treatment with Poteligeo to enable the identification of patients who would benefit from the drug. Poteligeo Test IHC is for use on tissue samples, such as lymph nodes, whereas Poteligeo Test FCM uses flow cytometry to analyze blood samples from patients.

16.7 Molecular Profiling of Cancer

Profiling of the 60 human cancer cell lines (the NCI-60) is being used by the NCI's Developmental Therapeutics Program (DTP) to screen >100,000 chemically defined compounds and natural product extracts since 1990. In statistical and

machine learning analyses, the screening data have proved rich in information about drug mechanisms of action and resistance. The NCI-60 panel already constitutes by far the most comprehensively profiled set of cells in existence, and much more molecular profile information on them is coming. The data have already yielded considerable biological and biomedical insight, but we have only scratched the surface thus far. The real value is realized when biomedical scientists with particular domain expertise are able to integrate and use the information fluently for hypothesis generation and hypothesis testing. Given the large drug activity database, the NCI-60 cell line panel provides a unique opportunity for the enrichment of pharmacologic hypotheses and for advances toward the goal of personalized medicine (Weinstein 2006).

16.8 Targeted Cancer Therapies

Targeted cancer therapy means selective action against molecular targets expressed in tumors. Conventional small-molecular therapeutics is usually targeted through selective action on the molecular machinery of the targeted cells. Targeted therapy also refers to screening patients so as to increase effectiveness of some form of therapy. Targeting reduces failure in both the drug development clinical research and postmarketing phases.

16.8.1 Selective Destruction of Cancer Cells

A problem with conventional chemotherapy or radiotherapy is that damage is not limited to cancer cells but involves normal cells as well. It is easy to kill cells in vitro and many new anticancer drugs are being discovered. However, it is difficult to selectively kill cancer cells in vivo without harming normal cells. Even though some success is achieved in animal experiments, it is difficult to translate these findings into practical management of cancer patients. Strategies for selective destruction of cancer in vivo are:

- Drugs for selective disruption of cancer metabolism: sphingolipids
- Hyperbaric oxygen (HBO) as adjunct to radiotherapy
- Genetically engineered bacteria for selective destruction of cancer
- Use of MAbs to selectively target anticancer agents to receptors on cancer cells
- Targeting response to transformation-induced oxidative stress
- Targeting enzymes to prevent proliferation of cancer cells: CFI-400945

16.8.1.1 Sphingolipids

Cancer cells are sensitive to nutrient limitation because cancer cell's ability to generate adenosine triphosphate (ATP) is compromised under these conditions.

In addition, most cancer cells have defects in autophagy, the catabolic process that provides nutrients from internal sources when external nutrients are unavailable. In contrast, normal cells can adapt to the nutrient stress that kills cancer cells by becoming quiescent and catabolic. A study has shown that FTY720, a water-soluble sphingolipid drug that is effective in many animal models of cancer, selectively starves cancer cells to death by downregulating nutrient transporter proteins (Romero Rosales et al. 2011). Consistent with a bioenergetic mechanism of action, FTY720 induced autophagy of cancer cells, but normal cells were protected. AAL-149, an FTY720 analog that lacks FTY720's dose-limiting toxicity, also triggered transporter loss and killed patient-derived leukemia cells while sparing cells isolated from normal donors. Because FTY720 analogs target the metabolic profile of cancer cells rather than specific oncogenic mutations, they should be effective against several tumor types, particularly in combination with drugs that inhibit autophagy.

16.8.1.2 Hyperbaric Oxygen

HBO, i.e., oxygen under higher than atmospheric pressure, is used for the treatment of several disorders. HBO has been investigated as an adjunct to radiotherapy of cancer. It is well recognized that hypoxia influences the response of cells and tissues to radiation and increases the resistance of cancer to radiotherapy requiring higher radiation doses that can normal tissues. HBO is considered to be the most effective method for counteracting tumor hypoxia for enhancing the effect of radiotherapy on cancer, but this approach has been shown to be effective in only some types of cancer, e.g., GBM (Jain 2009b). In spite of several studies, the controversy has not been resolved. Combination of antineoplastic agents and HBO induces dual injury to the mitochondrial respiration and cell membranes. HBO can be added to regimes combining radiotherapy with chemotherapy. Concomitant HBO enhances the effects of 5-fluorouracil on malignant tumors, but no clinical trials have been done to evaluate this combination.

16.8.1.3 Targeting Response to Transformation-Induced Oxidative Stress

Malignant transformation is often associated with enhanced cellular stress and DNA damage. Cancer cells adapt to this stress to survive and may become dependent upon non-oncogenes that do not ordinarily perform such a vital function in normal cells. Therefore, targeting this non-oncogene dependency may result in selective death of cancer cells. A cell-based small-molecule screening and quantitative proteomic approach led to the unbiased identification of piperlongumine, a small molecule that selectively kills cancer cells but not normal cells (Raj et al. 2011). Piperlongumine increases the level of reactive oxygen species (ROS) and apoptotic cell death in both cancer cells and normal cells engineered to have a cancer genotype, irrespective of p53 status, but it has little effect on normal cells. Significant antitumor effects were observed in mouse xenograft tumor models treated with

piperlongumine, but no toxic effects were observed in normal mice. Moreover, piperlongumine inhibits the growth of spontaneous breast cancers in mice. These findings show that ability a small molecule can selectively induce apoptosis in cells that have a cancer genotype by targeting a non-oncogene dependency acquired through the expression of the cancer genotype in response to oxidative stress induced by malignant transformation.

16.8.1.4 Targeting Enzymes to Prevent Proliferation of Cancer Cells

CFI-400945 has been designed by a team of scientists in Canada and China to specifically prevent the proliferation of cancer cells but not damage normal cells. It targets an enzyme called PLK4, which plays a critical role in cell division, especially in cancer cells. Cells in genomically unstable cancers can have scores more chromosomes than the 46 present in normal cells, and these malignant cells rely on PLK4 to be able to continue to proliferate out of control. Targeting this enzyme would prevent survival of these cells. Animal experimental studies have been completed, and the FDA permission to start human clinical trials is pending with expected go-ahead in the fall of 2013. Initial trial with the drug will be a study in patients with breast or ovarian cancers to determine a safe dose.

16.8.2 Targeting Glycoproteins on Cell Surface

The biochemical signature that distinguishes cancer cells from normal cells is often carried on the outside of the cell membrane in the form of glycoproteins. These cell surface proteins are decorated with sugar chains in distinctive arrangements (or epitopes) that serve as therapeutic targets (or antigens) for agents such as monoclonal antibodies. Carbohydrates are also promising candidates for cancer control because they are present on cell surface and act as identification tags, through which they can interact with their surroundings. Interfering with the normal cell recognition phenomenon using a small or large sugar molecule has been shown to block the progression of tumors by blocking angiogenesis, cell-to-cell matrix interactions, and tumor invasion.

16.8.3 Targeting Pathways in Cancer

The phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway presents an exciting new target for molecular therapeutics. PI3K/AKT pathway regulates a broad spectrum of cellular processes, some of which are necessary to maintain normal physiological functions and explain the toxicity of the drugs targeting the pathway. Elucidation of the precise function of the PI3K/AKT isoforms could promote the development of isoform-specific approaches to provide a selective action on

tumor cells. Inhibition of the PI3K/AKT pathway at multiple sites or a combination with inhibitors of different signaling pathways may allow the development of an acceptable therapeutic index for cancer management. Further, inhibition of the PI3K/AKT pathway combined with conventional chemotherapy or radiation therapy may provide a more effective strategy to improve patient outcome. As molecular therapeutics target the underlying defects in tumors, molecular diagnostics are required to identify patients with particular genetic aberrations in the pathway to enable personalized cancer treatment.

16.8.4 Targeted Personalized Anticancer Medicines in Clinical Use

There are several cancer therapies that are targeted to specific mutations or receptors in tumors and often accompanied by companion diagnostics to personalize their use. Marketed personalized anticancer medicines are shown in Table 16.3 and are described later in this chapter under organs involved.

16.9 Functional Antibody-Based Therapies

Functional antibodies are biological molecules that trigger death in cancer cells but not healthy cells. Functional antibodies target molecules carried on the outside of a cancer cell membrane known as antigens. These cell surface proteins are decorated with sugar chains in distinctive arrangements that can be used as targets for therapeutic monoclonal antibodies. Antigens can act as biochemical signatures or markers that distinguish a cancer cell from a normal cell and one person's cancer from another. The selection of antibodies of interest at ARIUS Research Inc. is based on the ability of the antibody to activate an antigen to selectively produce cancer cell death. ARIUS holds proprietary and patented technology that allows it to generate large numbers of functional antibodies at low cost. The core of ARIUS' technology platform is its high-throughput functional screens, which enable the company to rapidly identify and select MAbs that show superior cancer-killing ability. These MAbs, earmarked as potential drug candidates, are reserved for ARIUS' functional antibody library. The ARIUS approach to antibody development offers the following advantages:

- The process can produce multiple antibody drug candidates for any solid tumor cancer type.
- The antibodies are functional in and of themselves and are drug candidates at the very outset.
- Development is more flexible and less expensive because the antibodies are derived from patient tumors and do not rely on isolated target antigens that may have to be in-licensed.
- The antibodies can be used to discover novel cancer antigens, since they are not produced against predefined targets.

Table 16.3 Marketed anticancer personalized medicines

Medicine/company	Target	Companion diagnostic (company)	Indication
Cetuximab (Erbitux)/Merck KGaA	EGFR	TheraScreen K-ras Mutation Kit (DxS/QIAGEN) to exclude nonresponders	Squamous cell carcinoma (head and neck), colorectal carcinoma
Crizotinib (Xalkori)/Pfizer	ALK	ALK Break Apart FISH Probe (Vysis) to detect rearrangements of the ALK gene	Non-small-cell lung cancer
Dasatinib (Sprycel®)/Bristol-Myers Squibb	BCR-ABL	Not required	Chronic myelogenous leukemia
Erlotinib (Tarceva)/Roche	EGFR	Cobas EGFR Mutation Test (Roche)	Non-small-cell lung cancer
Gefitinib (Iressa)/AstraZeneca	EGFR	EGFR mutation test (DxS/QIAGEN) to identify patients most likely to benefit	Lung adenocarcinoma
Imatinib (Glivec)/Novartis	BCR-ABL, KIT	No companion test for leukemia, but c-KIT immunoassay for patients with gastrointestinal stromal tumors	Chronic myelogenous leukemia Gastrointestinal stromal tumor
Nilotinib (Tarceva)/Novartis	BCR-ABL	Not required	Chronic myelogenous leukemia
Panitumumab (Vectibix)/Amgen	K-ras	Home-brew tests for the K-ras mutation	Colorectal cancer
Sorafenib (Nexavar)/Bayer HealthCare	RAF, VEGF, KIT, FLT3	Measuring levels of biomarkers, VEGF and KDR (VEGFR2), to determine response to sorafenib treatment (Bayer)	Renal cell carcinoma Liver cancer
Trastuzumab (Herceptin)/Roche	HER2	PathVysion HER-2 breast cancer test kit (Vysis/Abbott)	Breast carcinoma
Vemurafenib (Zelboraf)/Roche	BRAF	Multiplex PCR-based diagnostic for the BRAF V600E gene (Roche)	Melanoma

ALK anaplastic lymphoma kinase

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The developmental tasks remaining are similar to classic antibody development pathways with the exception of finding the target for the newly formed functional anticancer antibody. Generally, a number of biochemical and proteomic approaches are taken for the identification of the target antigen. In addition, a number of validation studies for the antibody are performed including testing for recognition of human cancer, as well as specificity studies. The antibodies are studied in animal models of human cancer to determine its effectiveness in vivo. If the antibodies are found to be safe and effective, then they become candidate for clinical study. ARIUS has built a library of over 100 functional antibodies plus attendant protocols and a database. Three of these are in animal testing phase:

1. ARvitamab (ARH460-23) suppresses tumor growth with the following feature: (a) prevents metastases in human lung cancer models, (b) is compatible with and additive to cisplatin chemotherapy to improve disease-free survival, (c) recognizes a widely distributed tumor-associated antigen (TAA), and (d) is nontoxic in animal models. The putative target antigen for ARvitamab exhibits increased expression in many cancers including those involving breast, pancreatic, colon, and prostate, as well as nonepithelial cancers such as melanoma and lymphoma.
2. ARH460-16-2 displays cytotoxic activity in vitro against human breast cancer, colon cancer, and melanoma cell lines. More significantly, it showed almost complete suppression of tumor growth in a prophylactic breast cancer xenograft model while the antibody was being administered. Ongoing research and development is aimed at identifying and validating the ARH460-16-2 target, establishing the safety and specificity of the antibody, and producing a humanized form of the antibody for human clinical trials.
3. ARH460-22-1 has cytotoxic activity in vitro against human breast cancer, colon cancer, and melanoma cell lines. Ongoing research is aimed at identifying and validating the antibody target and showing antibody safety and specificity. A humanized version of this antibody is currently under development.

The long-term aim of targeted antibody therapy is to match multiple antibodies to different antigens on each patient's cancer cell, delivering multiple cancer-killing messages simultaneously. Personalized therapy will improve on targeted therapy by further reducing the risks of failed treatment and improving the likelihood of cure.

Genentech's anticancer drug Herceptin may be considered the first targeted antibody therapy in that it is only appropriate for use in patients who overexpress the Her2/neu antigen on the surface of their breast cancer cells.

16.10 Personalized Cancer Vaccines

There are several types of cancer vaccines, which include nucleic acid-based, MAb-based, and cell-based vaccines. Various types of cells are used including tumor cells and dendritic cells (DCs). Combination of different methods and genetic modification of cells are also used. Personalized vaccines may be antigen-specific or

tumor-derived, but patient-specific vaccines may be a combined approach. Most of the personalized cancer vaccines are cell-based and these were the earliest forms of personalized medicine (Jain 2010). A true personalized vaccine is one in which patient's own cells are used.

16.10.1 Antigen-Specific Vaccines

Antigen-specific approach may generate an antigen-specific response even when the tumor antigens are not known. Currently the scope of cancer immunization is limited because most of the vaccines have targeted antigens that are restricted to a subset of patients. Functional genomics and proteomics will enable molecular characterization of whole transcriptomes and proteomes of cancer cells, thereby also identifying potential new targets for cancer immunotherapy. Based on fundamental immunological knowledge, the most promising approach would be patient-tailored.

16.10.1.1 Active Immunotherapy Based on Antigen Specific to the Tumor

Active immunotherapy is focused on overcoming the limitations of the immune system and directing it to mount an attack against cancer cells. Activating the immune system begins with the selection and modification of a tumor antigen specific to the cancer (e.g., prostatic acid phosphatase found in ~95 % of prostate cancers), which is produced using recombinant DNA technology.

The lead product in this category is sipuleucel-T (Provenge™, Dendreon Corporation), which targets prostatic acid phosphatase. A proprietary technique is then used to isolate antigen-presenting cells taken from a cancer patient, which are combined with the modified antigen using Antigen Delivery Cassette™. The activated cells are then readministered to the patient to stimulate T cells to recognize and attack cancer cells that contain prostatic acid phosphatase. Sipuleucel-T has been approved by the FDA for the treatment of patients with early-stage and advanced prostate cancer. In clinical studies, patients typically received three infusions over a 1-month period as a complete course of therapy. Integrated results of two randomized trials demonstrate a survival benefit for prostate cancer patients treated with sipuleucel-T with a modest toxicity profile (Higano et al. 2009). A phase III clinical trial, IMPACT (IMmunotherapy for Prostate AdenoCarcinoma Treatment), found that sipuleucel-T reduced the risk of death by 22.5 % compared with a placebo. The treatment extended the lives of patients by 4–5 months and 33 % of patients with advanced disease were still alive 3 years after treatment with sipuleucel-T. Although sipuleucel-T is prostate specific, the underlying principle may be applicable to other cancers and it may be used in combination with chemotherapy. Several MAbs are in preclinical development, which are designed to recognize a specific antigen present on tumor cells but not on healthy cells and bind to that antigen to cause the death of the tumor cell. By this approach healthy cells

should not be affected, reducing or eliminating the harsh side effects of many conventional cancer therapies.

GlaxoSmithKline is developing MAGE (melanoma antigen gene)-A3, a tumor antigen-based patient-specific vaccine for melanoma and it has undergone phase II clinical trials. Distinct gene expression profiles have been identified on pretreatment biopsies that are associated with a positive or negative clinical outcome, and this might be useful as a predictive biomarker for clinical trials of melanoma vaccines (Gajewski et al. 2010). Phase II clinical trials have demonstrated a clinical benefit by postoperative vaccine with MAGE-A3 in NSCLC and in stage IV melanoma, which have led to the current phase III trials (Peled et al. 2009).

16.10.2 Tumor-Derived Vaccines

Although cancers may arise by common mechanisms, i.e., through mutations in genes implicated in cell transformation (i.e., p53, ras), they undergo additional random mutations in other genes. These mutations lead to the expression of foreign antigens, forming a molecular “fingerprint” that uniquely characterizes the patient’s tumor. Because mutations are generated randomly, the antigenic fingerprint of one person’s cancer can never be duplicated in another person’s cancer. Thus individual cancers within the same pathological category are antigenically distinct. This fundamental property requires that each patient’s immune system be trained to recognize that patient’s specific cancer. This is the basis of manufacture of cancer immunotherapeutic from each patient’s own tumor tissue. Another approach is to identify as many candidates as possible for tumor-associated T-cell epitopes in individual patients. Expression profiling of tumor and normal tissue can be performed to identify genes exclusively expressed or overexpressed in the tumor sample.

Mass spectrometry enables the characterization of several different major histocompatibility complex (MHC) ligands from the same tumor sample. Combining these two analytic tools, it is possible to propose several candidates for peptide-based immunotherapy. This integrated functional genomic approach can be used for the design of antitumor vaccines tailored to suit the needs of each patient.

Whole tumor cells of the patient, rendered safe by irradiation and mixed with an immunological adjuvant, were one of the earliest forms of personalized cell therapy. This approach avoids the need for tumor antigens to be identified before treatment and allows all of the relevant antigens to be included in the vaccine. Initial clinical studies showed the safety of this approach, with side effects mainly limited to local reactions at the site of the vaccine injection. Immunogenicity of tumor cell vaccines can be improved by transducing the tumor cell with genes that encode key components of the immune response, e.g., cytokines such as granulocyte–macrophage colony-stimulating factor (GM-CSF) and costimulatory molecules.

Most of the cancer vaccines are being developed in the commercial sector. Whole tumor vaccines have gone through clinical trials. None of the tumor cell vaccines are in the market in the USA.

16.10.2.1 FANG Vaccine

The FANG™ vaccine (Gradalis Inc.) is an autologous tumor-based product incorporating a plasmid expressing a well-established immune activator, GM-CSF, and a novel bifunctional short-hairpin RNAi (bi-shRNAi) targeting furin convertase, thereby downregulating endogenous immunosuppressive transforming growth factor (TGF)-β1 and TGF-β2. It is manufactured from a cell suspension derived from a portion of a patient's tumor removed during surgery. bi-shRNAi is introduced into the cells by electroporation. Cells are then incubated overnight, irradiated, frozen, tested, and released. Vaccine is shipped to the patient's clinic where doses are thawed and administered monthly by intradermal injection. FANG manufacturing is a straightforward 2-day cGMP process that is applicable to nearly all tumor types with no modification, and it does not require patients to undergo apheresis or other treatments except surgical tumor removal if indicated.

Results of a phase I study showed that treatment with FANG was safe and significantly increased survival in patients with advanced stage cancer compared to patients who received other forms of treatment (Senzer et al. 2012). The vaccine elicits a robust and lasting immune response, resulting in statistically significant prolonged survival in patients with advanced stage disease. Currently, FANG is being evaluated in several phase II trials in patients with advanced ovarian cancer, advanced melanoma, and advanced CRC with liver metastases. In addition, Gradalis has initiated a clinical program evaluating FANG in children with Ewing's sarcoma.

16.10.2.2 MyVax

MyVax® (Genitope Corporation) is an investigational treatment based on the unique genetic makeup of a patient's tumor and is designed to activate a patient's immune system to identify and attack cancer cells. It combines a protein derived from the patient's own tumor with an immunologic carrier protein and is administered with an immunologic adjuvant. The development of this immunotherapeutic approach has been limited by manufacturing difficulties. Genitope has developed a proprietary manufacturing process that overcomes many of these historical manufacturing limitations. A phase II trial found that immunization of follicular lymphoma patients with MyVax is safe and patients often mount tumor-specific immune responses (Timmerman et al. 2009). These results form the basis of a current pivotal phase III trial of MyVax in follicular non-Hodgkin lymphoma.

16.10.2.3 OncoVAX

OncoVAX® (Vaccinogen Inc.) is a vaccine from the patient's own tumor with or without fresh frozen bacillus Calmette–Guerin as an adjuvant. The cells are dissociated, irradiated to make them non-tumorigenic, and administered to the patient by three weekly injections, starting 4 weeks after surgery. A booster vaccination is

administered 6 months later. OncoVAX® is administered to patients with colon cancer after surgery to reduce recurrence and deaths. Results of a phase III clinical trial showed a significant improvement in 5-year overall survival and recurrence-free survival in stage II colon cancer and some benefits in stage III colon cancer (Hanna et al. 2006). This study was accepted by the FDA as supportive data for the next phase IIIb clinical trial where disease-free survival will be used as a clinical endpoint for the interim analysis.

16.10.2.4 Tumor Cells Treated with Dinitrophenyl

M-Vax (AVAX Inc.) is a vaccine based on modification of autologous tumor cells with the hapten, dinitrophenyl (DNP), which binds to molecules on the surface of cells and helps trigger immune responses. DNP-treated cancer cells are combined with an adjuvant, bacillus Calmette–Guerin, and the vaccine is injected intradermally into cancer patients. The patient's immune system is then better able to recognize, locate, and combat remaining cancer cells that may have metastasized to other areas of the body. It is these remaining cancer cells that, if left undetected and untreated, can potentially form additional cancerous tumors and eventually lead to death. Immune responses help the body determine which foreign proteins to attack. The ability of DNP to modify proteins and render them more easily identified as foreign to the immune system has been well documented over the past 30 years. Clinical trials have been conducted in stage III melanoma patients with a 5-year survival rate of 44 % as compared to 20–25 % with surgery alone (Berd 2004). This vaccine may help prevent cancer recurrence and increase the long-term survival rate of patients with other cancers as well. O-Vax is in phase II clinical trials for ovarian cancer.

16.10.2.5 Prophage

Prophage (vitespen, Agenus) is a patient-specific and tumor-specific therapeutic cancer vaccine, which contains the heat shock protein, gp96, and associated peptides that are purified from the patients' own tumor tissue (Wood and Mulders 2009). Following surgery to remove a part or whole of the tumor, the tissue specimen is shipped frozen to Agenus, which prepares the vaccine and sends it back for intradermal injection when the patient has recovered from surgery. It has been tested in numerous patients in multiple cancers in clinical trials and approved in Russia as Oncophage® for the adjuvant treatment of kidney cancer patients at intermediate risk for disease recurrence. It has orphan drug designation from the FDA as well as EMEA for kidney cancer and glioblastoma. Results of clinical trials of Prophage show that:

- It is well-tolerated.
- It elicits tumor-specific T-cell responses and innate immune response irrespective of tumor type.
- Efficacy is most significant in patients with early-stage disease and low tumor burden.

16.10.2.6 Melacine

Melacine melanoma vaccine was developed by Corixa Corporation (now acquired by GlaxoSmithKline) consisting of lysed cells from two human melanoma cell lines combined with an adjuvant that includes monophosphoryl lipid A and mycobacterial cell wall skeleton, both of which activate the human immune system. Melacine vaccine is approved in Canada but not in the USA. It is administered as a two-shot vaccination delivered as four 6-month cycles, each consisting of ten treatments followed by a 3-week rest. Patients who respond are maintained on long-term therapy.

A randomized phase III trial of Melacine plus low-dose interferon (IFN)-α2b in malignant melanoma had an effect comparable to standard high-dose IFN-α2b but with less toxicity (Ding and Wei 2007). Analysis of clinical benefit following completion of the data sweep in patients who were positive for the expression of either class I MHC HLA A2 or C3 genes continued to show a highly statistically significant clinical benefit of Melacine in terms of increased disease-free survival. Patients with these genes account for an approximate 60–70 % of all melanoma patients.

16.10.3 Patient-Specific Cell-Based Vaccines

16.10.3.1 Dendritic Cell-Based Vaccines

Dendritic cells (DCs), named after their long arms, comprise a system of leukocytes widely distributed in all tissues. DCs are derived from bone marrow progenitors and circulate in the blood as immature precursors prior to migration into peripheral tissues. Within different tissues, DCs differentiate and become active in the taking up and processing of antigens and their subsequent presentation on the cell surface linked to MHC molecules. Upon appropriate stimulation, DCs undergo further maturation and migrate to secondary lymphoid tissues where they present antigens to T cells and induce an immune response. Dendritic cells can be derived from $CD34^+$ precursors in response to GM-CSF and TNF and from monocytes cultured with GM-CSF and interleukin-4. DCs have the capacity to prime tumor-specific T-cell responses and are considered to be potentially effective vaccines for immunotherapy of cancer.

Various approaches for ex vivo loading of DCs with tumor-specific antigens include tumor-derived peptide/protein, RNA/DNA, necrotic tumor cells, chaperone proteins, exosomes, and/or tumor cell–DC fusion (Janikashvili et al. 2010). DCs may be administered intravenously, intradermally, subcutaneously, or by intranodal or intratumoral injection.

DCVax (Northwest Biotherapeutics) is a personalized therapeutic cancer vaccine manufactured from the patient's own DCs that have been modified to teach the immune system to recognize and kill cancer cells bearing the biomarker of patient's tumor. Published clinical trials of DC vaccine for high-grade glioma patients suggest favorable clinical outcomes with evidence of low toxicity in effective induction

of antitumor immunity correlating with clinical improvement (Wheeler and Black 2009). Preliminary reports on DCVax-Brain clinical outcomes seem to follow these trends. DCVax-Brain has been granted an orphan drug designation and received clearance from the FDA to commence a phase II clinical trial for GBM. DCVax-Lung has received clearance from the FDA for phase I trials.

Imetelstat (Geron Corporation's GRNVAC1) is a therapeutic cancer vaccine comprised of autologous DCs loaded ex vivo with telomerase reverse transcriptase (hTERT) mRNA. hTERT represents an attractive target for cancer immunotherapy because it is overexpressed in most human tumors. Imetelstat is injected intradermally from where the dendritic cells travel to the lymph nodes and instruct cytotoxic T cells to kill tumor cells that express telomerase on their surface. Results of the first completed phase I/II clinical trial of Imetelstat in metastatic prostate cancer patients showed that the vaccine was well tolerated with no major treatment-related toxicities (Su et al. 2005). In addition, telomerase-specific T-cell responses were generated in 19 of 20 subjects and vaccination was associated with a statistically significant increase in PSA doubling time and clearance of prostate cancer cells from the patients' blood, indicative of potential clinical response. Imetelstat is a potent and specific telomerase inhibitor and so far the only drug of its class in clinical trials (Röth et al. 2010).

Vaccines based on genetically modified dendritic cells. DCs, which have been generated in vitro and transduced with genes coding for tumor-specific antigens or pulsed with tumor-specific antigen or peptide, could be useful for induction of cytotoxic T-cell responses. Genetic engineering of DCs to express immunosuppressive or immunoregulatory molecules may provide a novel method to promote graft tolerance, reducing dependence on systemic immunosuppression.

Gene therapy techniques can be applied to DC vaccines using recombinant nonreplicating viral vectors to provide efficient and reliable means of gene transfer. Genetic material is introduced into DCs to provide them a renewable source of antigen for presentation; this should lead to more sustained expression of antigen. The expression of viral (and therefore foreign) genes may boost the immune response, but this antiviral immunity primed by DCs may cause the immune system to destroy DCs rapidly in subsequent rounds of immunization. One solution may be to use viral vectors that do not result in the expression of viral genes, such as retroviruses or "gutless" adenoviral vectors. Adeno-associated viruses can be used to transduce human DCs and their main advantage is a decrease in viral-derived epitopes leading to decreased immunogenicity of the vector.

Lentivirus vectors can be used for genetic modification of human DCs and they have an advantage over retroviral vectors that they do not require target replication for efficient transduction. Approaches facilitating generation of DC vaccines for clinical trials and enhancing their viability, biodistribution, and capacity to stimulate antigen-specific immune responses are critical for immunotherapy. In one study, mouse bone marrow cells were programmed with lentiviral vectors so that they produced GM-CSF and IL-4 in an autonomous manner (Koya et al. 2007). Mice vaccinated with genetically modified DCs self-differentiated in vitro or in vivo

and produced potent antigen-specific responses against melanoma, which correlated with protective and long-term therapeutic anticancer effects. Thus, DC precursors can be genetically engineered after a single ex vivo manipulation, resulting in DC vaccines with improved activity.

Fusion of tumor cells with dendritic cells to produce cancer vaccines. In this approach, a product is created using a technique that fuses the patient's own tumor cells with powerful, immune-stimulating DCs. The fusion product is then injected back into the patient with the goal of sparking a specific immune response against the cancer. This individualized cell therapy presents the full complement of antigens specific to the patient's tumor.

The BiovaxID™ (Accentia BioPharmaceuticals) cancer vaccine evokes the power of each patient's immune system and primes it to recognize and eliminate malignant lymphoma cells, while sparing normal B cells. In this individualized therapy, cells are harvested from a patient's lymph node, and the unique cancer biomarkers on the outside of their cancer cells are identified. To create this idiotype vaccine, the antigen-bearing tumor cells are fused to antibody-producing mouse cells that act as mini-factories, churning out large quantities of the protein antigens, which are then given back to patients with an immune system booster. By priming the immune system with this antigen in the form of an autologous vaccine, the vaccine induces an immune response against the cancerous cells and creates an immune memory. Because it is derived from individual patient's cancer cells, the vaccine is a true targeted, personalized therapy. The vaccine's anticancer effect is different than nontargeted traditional therapy, as it arises from the immune system's defense cells' innate ability to selectively target foreign antigens. Moreover, the immune response triggered by the vaccine against the cancerous tissue is a natural disease-fighting mechanism and is associated with minimal toxicity. Phase I and II clinical trials demonstrated the immunogenicity, safety, and therapeutic efficacy of BiovaxID (Reinis 2008). It is in phase III clinical trials at MD Anderson Cancer Center (Houston, TX) for follicular lymphoma.

Concluding remarks about DC-based vaccines. DC-based cancer vaccines are a major focus in cancer immunotherapy as the primary functions of DCs are the initiation and regulation of immune responses. However, some tumors may stop responding to DC-based vaccines due to the development of immune tolerance, which can be overcome by personalized DC-based cancer vaccines as they contain nearly all the antigens in a tumor. Combination with chemotherapy may also be helpful by elimination of cancer cells and inhibition of inhibit tumor-induced suppressive factors.

16.10.4 Adoptive Cell Therapy

Adoptive cell therapy (ACT), also called adoptive immunotherapy, is the isolation of antigen-specific T lymphocytes, their ex vivo expansion and activation, and subsequent administration in large numbers to the autologous host. It is a promising

approach to inducing antitumor immune responses. The molecular identification of tumor antigens and the ability to monitor the persistence and transport of transferred cells have provided new insights into the mechanisms of tumor immunotherapy. Several studies have shown the effectiveness of ACT for the treatment of patients with selected metastatic cancers. Important features of studies on this topic are:

- Preclinical models have identified characteristics of lymphocyte cultures that are required for successful ACT.
- The most important characteristic is the presence of high-affinity, tumor antigen-specific $CD8^+$ T cells. There is generally a direct correlation between treatment efficacy and the number of transferred, tumor-specific cells.
- Preclinical models have also identified ways to manipulate the host immune environment that increase ACT therapeutic efficacy. These include immunosuppression before cell administration and concurrent IL-2 administration with the transferred T cells.
- Lymphocyte cultures that were selected for reactivity against melanoma antigens, including melanocyte-differentiation antigens, mediated cancer regression in some patients with metastatic melanoma. Melanoma-reactive cultures that were suitable for ACT were generated from tumor-infiltrating lymphocytes (TILs) that were rapidly expanded with anti-CD3 antibody.
- The generation of tumor antigen-specific lymphocyte cultures is evolving rapidly, spurred on by the identification of tumor antigens and the T-cell receptors that recognize them.

Further improvements to ACT will depend on a deeper understanding of the basic immunological processes, including the role of $CD4^+$ T cells in the antitumor inflammatory response, the ability of lymphocytes to persist in vivo and travel to tumors, and the mechanisms of ACT augmentation by previous host immunosuppression.

ACT regimen results in the in vivo expansion and enhanced activity of these cytotoxic lymphocytes. To date, of the 35 melanoma patients treated by ACT in a phase II clinical trial, 18 patients (51 %) achieved an objective response, with three patients exhibiting a complete response (CR) (Dudley et al. 2005). NCI has identified and characterized a number of melanoma TAAs, including gp100 and MART-1, and has developed a lymphodepleting nonmyeloablative regimen used for ACT. Transgene and the NCI have collaborated to evaluate new candidate cancer vaccines, with the objective to assess the boosting effect of the vaccination on the lymphocytes' activity. These novel vaccines were designed by Transgene using viral vectors to express melanoma antigens. Such a vaccination has already demonstrated increased in vivo clonal expansion and maintenance of adoptively transferred tumor antigen-specific cytotoxic lymphocytes in preclinical models. The NCI will conduct preclinical evaluation of the vaccines and sponsored a phase I/II trial. The adoptive transfer of in vitro-generated tumor antigen-specific cytotoxic T lymphocytes (CTLs) provides a promising approach to the immunotherapy of cancer. A phase I study was conducted to test the feasibility, safety, and survival of adoptively transferred Melan-A-specific CTL lines in melanoma patients and shown to induce clinical tumor-specific immune responses without major adverse effects (Mackensen et al. 2006).

TILs already appear to offer significant patient benefit and this approach now warrants further development. Genetically engineered T cells offer a means to endow peripheral blood T cells with antitumor activity and in principle these techniques could allow the treatment of a wide range of cancers. Genetic engineering also offers the means to endow T cells with new properties and enhanced functions. Proof-of-principle trials have shown clear responses with T-cell receptor-engineered T cells and this can be built on with further development (Hawkins et al. 2010).

Epstein–Barr virus (EBV) infection is associated with a heterogeneous group of tumors, including lymphoproliferative disorders, Hodgkin disease, nasopharyngeal carcinoma, and Burkitt's lymphoma. As these cancers express viral antigens, they can be treated by ACT strategies relying mostly on in vitro generation and expansion of virus-specific CTL, which can be administered to patients for both prophylaxis and treatment. ACT with EBV-specific CTL is safe, well-tolerated, and quite effective in the case of most immunogenic tumors, e.g., posttransplant lymphoproliferative disease (Merlo et al. 2008).

16.10.4.1 Combination of Antiangiogenic Agents with ACT

Although ACT-based immunotherapies can achieve cancer regression in animal models and in up to 70 % of patients with metastatic melanoma, it is possible that the tumor vasculature impedes the egress of tumor-specific T cells, thus hindering immunotherapy. Disruption of the proangiogenic interaction of VEGF with its receptor VEGFR-2 has been reported to "normalize" tumor vasculature, enhancing the efficacy of chemotherapeutic agents by increasing their delivery to the tumor. Administration of an antibody against mouse VEGF synergized with ACT to enhance inhibition of established, vascularized, B16 melanoma and improved survival (Shrimali et al. 2010). Anti-VEGF antibody significantly increased infiltration of transferred cells into the tumor. Thus, normalization of tumor vasculature through disruption of the VEGF/VEGFR-2 axis can increase extravasation of adoptively transferred T cells into the tumor and improve ACT-based immunotherapy. These studies provide a rationale for the exploration of combining antiangiogenic agents with ACT for the treatment of patients with cancer.

16.10.4.2 Genetically Targeted T Cells for Treating B-Cell Malignancies

Human T cells targeted to the B-cell-specific CD19 antigen through retroviral-mediated transfer of a chimeric antigen receptor (CAR) called 19z1 have shown significant but partial in vivo antitumor efficacy in an ALL model (Brentjens et al. 2007). The causes of treatment failure in this model were investigated and approaches were designed to enhance the efficacy of this adoptive strategy. Expression of the 19-28z CAR, containing the signaling domain of the CD28 receptor, enhanced systemic T-cell antitumor activity when compared with 19z1 in treated mice. T-cell injections, designed to prolong in vivo T-cell function, further improved long-term survival.

Thus combined in vivo costimulation and repeated administration enhance eradication of systemic tumor by genetically targeted T cells. The finding that modifications in CAR design as well as T-cell dosing enable the complete eradication of systemic disease affects the design of clinical trials using this treatment strategy. The investigators have an ongoing study using these T cells in CLL and are planning a trial in patients with ALL. The idea is that a patient's own T cells are taken and reeducated by inserting a gene into them that will enable them to produce a receptor to recognize B-cell cancers, and then they are returned to the patient where they should be able to attack and kill the tumor cells. Because the technique uses a patient's own T cells, there is little risk of compatibility issues or rejection, as there might be with human stem cell transplant. Human stem cell transplant, following radiation or chemotherapy, is currently incorporated into the treatment of several B-cell malignancies.

The extensive exploitation of the antitumor effect of donor lymphocytes infused after allogeneic hematopoietic stem cell transplantation (allo-HSCT) is limited by the risk of graft versus host disease (GVHD). To overcome this limitation, the therapeutic potential of donor lymphocytes engineered with the suicide gene thymidine kinase (TK) of herpes simplex virus (HSV) was investigated in patients experiencing recurrence of hematologic malignancies after allo-HSCT (Ciceri et al. 2007). The antitumor effect tightly correlated with the in vivo expansion of TK^+ cells. Immunization against HSV-tk was observed in some patients but did not preclude an effective GvL. These data validate the feasibility, safety, and efficacy of TK^+ cells in the context of allografting and represent the basis for a broader application of this technology. This technology is being clinically developed by MolMed.

16.10.4.3 Genetic Engineering of Tumor Cells

Many companies have effective vaccines for stimulating killer T lymphocytes. The missing link is making good vaccines for helper T lymphocytes. That problem has been solved by Antigen Express scientists, who developed means to suppress the expression of a specific immunoregulatory protein (Ii). This protein can block antigens from stimulating T helper cells. By inhibiting this protein, a whole range of antigens from tumors can now be recognized by T helper cells, greatly boosting the immune response to cancer.

16.10.4.4 Hybrid Cell Vaccination

The hybrid cell vaccination approach to cancer immune therapy aims at the induction of tumor-specific cytotoxic T cells and was developed for the following purposes:

- To recruit and activate T-cell help for the induction of tumor-specific cytotoxic T cells
- To correct defects in costimulatory signaling
- To utilize a large number of unidentified TAAs
- For individualized therapy that can be applied instantly without long preparation

Hybridoma technology involves the selection of long-term lines on the basis of their resistance to anticancer drugs and according to specific functions desired. The fusion partners are of the same tissue origin and are controlled by similar genetic programs. The vaccines are irradiated prior to inoculation to ensure that the tumor does not grow and spread in the body.

Clinical trials of hybrid cell vaccination have been performed in patients suffering from malignant melanoma or renal cell carcinoma and cases of complete remission have been reported. The side effects seen in these trials were those of induced immune response. Hybrid cell vaccination is a feasible strategy for the treatment of cancer and is well suited for individualized therapy. Future trials will establish the criteria for the selection of patients and the malignancies suitable for this therapy.

16.10.5 Personalized Peptide Cancer Vaccines

Following the identification of TAAs in different tumor histotypes, many vaccination strategies have been investigated, including peptide-based vaccines. Results of the first decade of clinical experimentation, although demonstrating the feasibility and the good toxicity profile of this approach, provided evidence of clinical activity only in a minority of patients despite inducing immunization in up to 50 % of them. Different approaches have been developed recently in order to induce stronger peptide-induced immune-mediated tumor growth control, possibly translating into improved clinical response rates, with specific focus on multipeptide-based anticancer vaccines (Pilla et al. 2009). This strategy offers many advantages, such as the possibility of bypassing tumor heterogeneity and selection of antigen-negative clones escaping peptide-specific immune responses or combining HLA class I- and class II-restricted epitopes, thus eliciting both CD4- and CD8-mediated immune recognition. Notably, advances in antigen discovery technologies permit further optimization of peptide selection, in terms of identification of tumor-specific and unique TAA as well as antigens derived from different tumor microenvironment cell components. With the ultimate goal of combining peptide selection with patient-specific immunogenic profile, peptide-based anticancer vaccines remain a promising personalized treatment for cancer patients, as shown by preclinical and clinical studies. The use of personalized peptide vaccination combined with chemotherapy has been explored for cancer patients, e.g., those with breast and prostate cancers, which are described under the cancers of respective organs in a following section.

16.10.6 Current Status and Future Prospects of Personalized Cancer Vaccines

This chapter has identified some of the important technologies and given examples of their application. The review of current state of technologies relevant to cancer indicates good prospects for the development of personalized cancer therapies.

Some of the current clinical trials of personalized cancer vaccines are shown in Table 16.4.

Numerous other clinical trials of cancer vaccines have been conducted with a high failure rate. The reasons for failure include the following:

- The immune system is already damaged by chemotherapy in some patients and may not respond to vaccines.
- Vaccines based on a single antigen are less effective than those that raise an immune response against a broad range of tumor antigens to minimize the chance of the tumor becoming resistant to the therapy.
- Immune response to vaccine may take a few months to evolve and tumors that grow rapidly may outpace it.
- Some cancer patients with advanced and bulky tumors are not good subjects for immunotherapy.

16.11 Personalized Radiation Therapy

Radiation therapy is the most common agent used in cancer therapy and up to 60 % of cancer patients receive it. However, physicians are unable to distinguish the differences in radiosensitivity across tumors when prescribing it. Accurate prediction of human tumor response to radiation therapy and concomitant chemoradiation would be an important tool to assist the physician in making recommendations for tumor treatment. Most studies that define the molecular biomarkers for the prediction of radiation response are based on the observation of gene expression using immunostaining, Northern blot, or Western blot analysis of a single or several genes. The results vary among the different studies and some results are contradictory. However, these studies agree that the change in expression of the tumor-related gene affects the radiation response. A novel approach was developed to predict the radiation response of human tumor using Atlas™ human cancer 1.2 cDNA array to analyze the expression profile of 1,187 tumor-related genes in radiation-resistant and radiation-sensitive tissues (Hanna et al. 2001). Sixty tumor-related genes were selected as predictors of radiation response of squamous cell carcinoma of the head and neck. Using the expression intensity of these 60 tumor-related genes, in combination with cluster analysis, researchers have introduced a mathematical method that successfully predicted the radiation identity of two tumor samples.

Radiation therapy treatment comes with serious side effects in 5 % of patients. Some cases of toxicity are associated with abnormal transcriptional responses to radiation. Screening blood for the activity level of 24 genes can identify those patients most likely to react badly to radiation (Rieger et al. 2004). This tool may help physicians to tailor treatments for individual patients. Some factors are a tip-off that a patient may have an unusually severe reaction to radiation. Patients who have autoimmune diseases such as diabetes or lupus or who have certain rare genetic diseases need to be monitored carefully or avoid radiation altogether. Even beyond these obvious signs, some patients suffer disfiguring, disabling, or extremely painful effects. These may include wounds that do not heal, skin burns so severe that

Table 16.4 Clinical trials of personalized cancer vaccines

Vaccine	Sponsor	Description/indication	Phase/status
AGS-003 autologous DC vaccine	Argos Therapeutics	Arcelis™ personalized immunotherapy: DCs pulsed with amplified mRNA from the patient's tumor/metastatic renal cell carcinoma	Phase II
BiovaxID®	Biovest International Inc.	Patient-specific active immunotherapy/mantle cell lymphoma and follicular non-Hodgkin lymphoma	Phase II/III Compassionate use in Europe
CVac™: dendritic cell (DC)/autologous vaccine	Prima Biomed	DCs + mannan fusion protein (adjuvant mannan, attached to a tumor cell surface protein, mucin 1)/ ovarian cancer	Phase IIb
DC/autologous vaccines	Northwest Biotherapeutics	1. DCVax®-Prostate/cancer of prostate 2. DCVax®-Brain/glioblastoma multiforme	1.Phase III/USA 2. Phase II/USA
FANG™ vaccine	Gradalis Inc.	Autologous tumor cell vaccine expresses rhGM-CSF and the bifunctional RNAi effector, bi-shRNAfurin	Phase II
HER2/neu hybrid vaccine	Antigen Express	Peptide immunotherapeutic/HER2/neu-positive breast cancer	Phase II
Imetelstat (GRNVAC1)	Geron Corporation	Autologous DCs loaded ex vivo with telomerase reverse transcriptase/metastatic prostate cancer	Phase II
MGN1601	Mologen	Gene-modified tumor cells based on MIDGE (Minimalistic Immunogenically Defined Gene Expression) vectors/kidney cancer	Phase I
MyVax®	Genitope Corporation	Designed to activate a patient's immune system to identify and attack cancer cells/follicular non-Hodgkin lymphoma	Phase III

Oncophage (now called Prophage, except in Russia)	Agenus Inc.	Renal cancer and malignant melanoma	Phase III in the USA Marketed in Russia
OncoVAX	Vaccinogen Inc.	Vaccine from the patient's own tumor cells, which are irradiated to make them noncarcinogenic/colorectal cancer	Phase III
OVAX autologous cancer cell vaccine	AVAX Technologies Inc.	Haptenization, i.e., chemical modification of antigens on the cancer cell to stimulate the patient's immune system/relapsed stage III–IV ovarian cancer	Phase II
Personalized idiotype vaccine	Bayer Innovation GmbH	Vaccine antigen is produced in tobacco plants based on magnICON® technology	Phase I
Stimuvax® DC vaccine	Oncothyreon Inc./Merck KGaA	Elicits T-cell-mediated immune response to cancer cells expressing the target MUC1/metastatic NSCLC	Phase III
TroVax: dendritic cell vaccine	Oxford BioMedica	Vaccinia virus Ankara is engineered to deliver the 5T4 antigen to destroy cancer cells/TRIST (TroVax Renal Immunotherapy Survival Trial) in advanced and metastatic renal cell carcinoma	Phase III

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they require plastic surgery, or brain damage. Past attempts to identify these patients by screening the cancer cells themselves have failed. Screening blood rather than cancer cells means the test would be more accessible to patients. Patients who respond poorly to radiation might have cells that do not properly recognize or repair radiation-induced DNA damage. These cells may turn on different genes, or the same genes at different levels, compared with normal cells exposed to radiation. Knowing which patients may have severe radiation toxicity could make treatment decisions easier. For cancers of the breast or prostate, surgical options can be as effective as radiation. For other cancer patients, radiation may be the best treatment. However, patients at risk for high toxicity may also have cancers that die in response to much lower radiation doses. In such cases, radiation—though at greatly reduced doses—may still be an option. Even those patients who do not have severe radiation toxicity may also benefit from this study. If you eliminate those patients with toxicity are excluded, the remaining patients may be eligible for higher doses. If patients are treated individually rather than as averages, many could receive higher, more effective doses. Before personalized radiation treatment becomes possible, investigators must validate the 24-gene test on a larger number of patients. Then the screen needs to be commercialized to make it available to medical laboratories.

Genetic profiles of tumor response to treatment techniques available could help physicians prescribe radiation therapy customized for individual cancer patients' needs. An important finding is that a trio of proteins often present in cancer cells—natural killer (NK)-κB, extracellular signal-regulated kinase (ERK), and GADD45β—protect the tumor from destruction by radiotherapy and might lead to radioresistance. These proteins are coactivated by ionizing radiation in a pattern of mutually dependence to increase cell survival and defend cells against the cytotoxicity induced by ionizing radiation (Wang et al. 2005). Administration of drugs that block the proteins would enable irradiation of the cancer with lower radiation doses. This would be not only more effective against the cancer but also less harmful to the patient. A deeper understanding of the relationship among these protein molecules, gained through genetic testing, would be the key to a successful attack on cancer. If one can test cancer cells not just for three proteins but for thousands, the "genetic fingerprint" such a test would provide might help the formulation of better therapies to destroy cancer.

16.11.1 Use of Radiation Sensitivity Biomarkers to Personalized Radiotherapy

A systems biology understanding of radiosensitivity has been used for identifying radiation-specific biomarkers (Eschrich et al. 2009). The authors used radiosensitivity modeling, as represented by the survival fraction at 2 Gy, in 48 human cancer cell lines. A linear regression algorithm was applied for integrating gene expression with biological variables, including ras status (mut/wt), tissue of origin, and p53 status. The biomarker discovery platform is a network representation of the genes by linear regression analysis. This network of top 500 genes identified by this approach was reduced to a ten-hub network that includes c-Jun, HDAC1, RELA

(p65 subunit of NFKB), PKC-beta, SUMO-1, c-Abl, STAT1, AR, CDK1, and IRF1. Nine targets associated with radiosensitization drugs were linked to the network, demonstrating clinical relevance. Furthermore, the model identified four significant radiosensitivity clusters of terms and genes. Ras was a dominant variable in the analysis, as was the tissue of origin, and their interaction with gene expression but not p53. Overrepresented biological pathways differed between clusters but included DNA repair, cell cycle, apoptosis, and metabolism. The c-Jun network hub was validated using a knockdown approach in human cell lines representing different cancers. This novel radiation-biomarker discovery platform, using a systems biology modeling approach, will play a central role in the integration of biology into clinical radiation oncology for personalizing therapy. It has been clinically validated in rectal cancer, esophageal cancer, head and neck cancer, and breast cancer (Eschrich et al. 2012). Such a molecular assay of tumor radiosensitivity is a road map toward biology-based personalized radiation therapy (Torres-Roca 2012). CvergenX, in collaboration with the Moffitt Cancer Center, is commercializing this assay.

16.12 Role of Nanobiotechnology in Personalized Management of Cancer

The role of nanobiotechnology in cancer has been described in Chap. 9 (Nanooncology). Nanodiagnostics has the potential to improve early diagnosis of cancer. Nanobiotechnologies will also improve the detection of cancer biomarkers as basis for devising diagnostics in combination with therapeutics. Some examples of application of nanobiotechnology in improving cancer management are as follows:

- Paramagnetic nanoparticles for targeted delivery to tumors, diagnosis by MRI, and destruction of tumors by localized thermal therapy.
- Dendrimers are a novel class of 3D nanoscale, core–shell structures that can be precisely synthesized for a wide range of applications including oncology. Polyvalent dendrimers interact simultaneously with multiple drug targets. They can be developed into novel targeted cancer therapeutics. Dendrimers can be conjugated to different biofunctional moieties such as folic acid using complementary DNA oligonucleotides to produce clustered molecules, which target cancer cells that overexpress the high-affinity folate receptor.

16.13 Design of Future Cancer Therapies

A better understanding of cancer biology would enhance the design of future therapies for cancer. For example, PCR can already be used to assess the efficacy of new therapies for leukemias. Future targets for cancer therapies may include defective proto-oncogenes or the tumor suppressor genes themselves. A gene therapy strategy might be employed to correct or replace the defective gene. In cancers with multifactorial etiology, it may be possible to interrupt one or two steps in the complex pathways, thereby hindering the overall evolution of the tumor. Serial analysis of

gene expression (SAGE) studies have demonstrated that tumor and normal endothelium are distinct at the molecular level, a finding that may have significant implications for the development of antiangiogenic therapies.

The use of emerging technologies in early clinical trials is enabling quick assessment of the efficacy of anticancer agents and rational drug development rather than a "trial-and-error" method. The identification of specific biomarker molecules in tumor tissue will allow the prediction of clinical outcomes in response to drug treatment. Such biomarkers can be detected by a variety of techniques including IHC, microarrays, and qPCR. The cancer clinical trial toolkit, including biomarkers that can detect antitumor activity of anticancer agents, can guide patient selection for specific drug treatments.

16.13.1 Role of Epigenetics in Development of Personalized Cancer Therapies

Epizyme Inc. is focused on discovering novel, small-molecule drugs that act as selective inhibitors of key epigenetic enzymes. The selective addition of methyl groups to specific sites on the histones is controlled by the action of a unique class of enzymes known as the histone methyltransferases (HMTs). Once the methyl group has been deposited on the histone site, the affected genes continue to be regulated (turned on or off) until this chemical unit is removed by other enzymes, known as histone demethylases. In a like manner, other enzyme classes can decorate DNA and histones with other chemical species and still other enzymes can remove these species to provide temporal control of gene regulation. The strategy at Epizyme Inc. is to target the HMTs as a family of *S*-adenosylmethionine-utilizing enzymes, making full use of lessons learned from kinases as drug targets and exploiting technological platforms that allow parallel processing of multiple enzymes of similar mechanism. Two programs at Epizyme are based on epigenetics: DOT1L targeting for the treatment of mixed lineage leukemia and EZH2 targeting for the treatment of certain non-Hodgkin lymphomas and breast cancer subtypes. Both of these programs are at subclinical stage.

Aberrant epigenetic modifications are frequently associated with distinct cancer types and have potential utility as biomarkers. The development of DNA methylation biomarkers that are predictive of a response to chemotherapy, however, is still in its infancy. Several studies have reported associations between DNA methylation biomarkers and response to chemotherapy.

16.13.2 Personalized Therapy of Cancer Based on Cancer Stem Cells

Cancers may rely on "cancer stem cells" that share the self-renewal feature of normal stem cells. CSCs form new tumors and may not be eliminated by current therapies. This has changed the perspective with regard to new approaches for treating cancer.

CSCs are slow dividing and inherently drug-resistant, and their eradication would be necessary for long-term success in cancer treatment. The CSC concept could be used to better tailor treatment strategies to individual patients. Most traditional anti-cancer agents affect primarily bulk tumor cells by disrupting their proliferation and/or survival. Even the newer "targeted" agents, such as receptor TKIs and some MAbs, though a considerable improvement over older agents, are still largely aimed at proliferation, survival, and angiogenesis pathways that may or may not affect the stem cell population. CSCs are less likely to be killed than bulk tumor cells by these approaches. Improved methods will be required to identify, isolate, and genetically profile the stem cell population in cancers from individual patients. CSCs, amplified from individual clinical specimens, should be tested for gene expression profiles and sensitivity to a battery of agents, leading to individualized decisions on the selection of the best therapeutic strategies. The antineoplastic agents of the future will have to target the ancient developmental molecular pathways on which stem cells depend on for replication and survival. Thus, an improved understanding of these pathways and their roles in CSCs could lead to a new generation of more selective and effective antineoplastic treatments (Song and Miele 2007).

16.14 Role of Oncoproteomics in Personalized Therapy of Cancer

Clinical proteomics is an exciting new subdiscipline of proteomics that involves the application of proteomic technologies at the bedside, and cancer, in particular, is a model disease for studying such applications. Oncoproteomics is the term used for application of proteomic technologies in oncology. Proteomic technologies are being developed to detect cancer earlier, to discover the next generation of targets and imaging biomarkers, and to tailor the therapy to the patient. Proteomic technologies will be used to design rational drugs according to the molecular profile of the cancer cell and thus facilitate the development of personalized cancer therapy. Proteomic separation and analytical techniques are uniquely capable of detecting tumor-specific alterations in proteins.

16.14.1 Cancer Tissue Proteomics

Cancer tissue proteomics implies direct tissue profiling and use of imaging MALDI MS to provide a molecular assessment of numerous expressed proteins within a tissue sample. Analysis of thin tissue sections results in the visualization of 500–1000 individual protein signals in the molecular weight range from 2,000 to over 200,000 (Chaurand et al. 2004). Laser capture microdissection (LCM), in combination with MS, enables the acquisition of protein signatures from a single cell type within a heterogeneous sample. These signals directly correlate with protein distribution

within a specific region of the tissue sample. The systematic investigation of the section allows the construction of ion density maps, or specific molecular images, for virtually every signal detected in the analysis.

MALDI-TOF (Time of Flight) MS can be used to generate protein spectra directly from frozen tissue sections from surgically resected cancer specimens. Profiling MALDI MS has been used to monitor alterations in protein expression associated with tumor progression and metastases. Current data suggests that MALDI MS will be superior to immunohistochemical stains and electron microscopy in identifying the site of origin for tumors currently labeled as "tumor of unknown primary." Another application in surgical pathology would be the rapid evaluation of margins of surgical excision of a tumor. Routine analysis of surgical margins by frozen section is very difficult because some cancers invade in a single-cell fashion without producing a grossly identifiable mass. Sensitivity of MS enables the detection of even a few tumor cells within a significantly larger portion of tissue.

The capability of MALDI MS to measure susceptibility and response to therapeutic agents in tumor and surrounding tissues is particularly useful in personalized management of cancer. The original protein profile obtained from the primary tumor can be used to influence the selection of therapeutic agents. The levels of chemotherapeutic agents can be measured directly from a tissue biopsy to assess adequacy of delivery to a particular organ site. It will also help in detecting alterations in specific molecular pathways directly modulated or indirectly affected by the anticancer agent. Finally, it could be used to monitor chemotherapy effects on the tumor.

16.15 Role of Sequencing in Personalized Therapy of Cancer

Discoveries made through application of the human genome sequencing have already an impact on practice of oncology and have influenced the design of clinical trials for new cancer therapies. Sequencing the entire TP53 gene from various types of cancer using NGS with ultradeep coverage has enabled a curated mutation database for TP53 mutations and a framework for mutation database analysis (Edlund et al. 2012). Such databases are expected to play central roles in personalized medicine by providing targets for drug development and biomarkers to tailor treatments to each patient.

In the future, research into cancer genomes will expand, and cooperative global initiatives will generate full-genome sequences of various cancers, yielding complete catalogs of somatic mutations in each one. These studies will reveal essentially the full repertoire of mutated cancer genes, enabling us to determine how many and what combinations of mutated cancer genes are necessary to generate an individual cancer. Sequencing will evolve from a research tool to cancer diagnostic. The rapid development of NGS technologies seems likely to be transformative. Within a few years, a complete cancer genome sequence will be obtainable for a few hundred dollars. It will be important to incorporate analysis of the genome and transcriptome

more widely into clinical trials in order to exploit the full clinical potential of information within the cancer genome and generating new and unexpected predictors of drug responsiveness and prognosis to enable personalized management of cancer (McDermott et al. 2011).

16.16 Pharmacogenomic-Based Chemotherapy

16.16.1 Whole-Genome Technology to Predict Drug Resistance

Millennium, a Takeda Oncology Company, uses whole-genome technologies, including gene and protein expression data, to predict the potential sensitivity or resistance of an individual patient's tumor to a single or group of drugs. The proteasome inhibitor, Velcade™ (bortezomib), approved for relapsed and refractory myeloma patients represents the first anticancer agent to include pharmacogenomic (PGx) assessments during its clinical development. PGx analyses of bone marrow samples using bioinformatic algorithms indicate there are significant differences in gene expression profiles, which may predict patients likely to respond to Velcade and those likely to be refractory to treatment. These PGx analyses also show promise in their ability to detect the relevant biological pathways associated with disease progression and the mechanism(s) associated with drug resistance.

The mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) encodes for a multifunctional receptor involved in lysosomal enzyme trafficking, fetal organogenesis, cytotoxic T-cell-induced apoptosis, and tumor suppression. M6P/IGF2R loss of heterozygosity (LOH) predicts poor therapeutic outcome in patients treated with radiotherapy alone. It also indicates that head and neck cancer patients with M6P/IGF2R allelic loss benefit most from chemotherapy added to radiotherapy.

16.16.2 Anticancer Drug Selection Based on Molecular Characteristics of Tumor

Cancer cells have defects within their systems related to the control of the cell cycle. These modifications may, however, confer selective sensitivity to appropriately designed drug therapy. Thus, molecular defects could potentially be linked to specific drug sensitivities. Such correlations might guide the selection of drugs for therapy based on the molecular characteristics of individual tumors. An example is the treatment of breast cancer with TCH (Herceptin; Genentech, USA), a humanized monoclonal antibody against the HER2 receptor. Overexpression of HER2 may occur as a somatic genetic change in breast cancer and other tumors. This correlates with poor clinical prognosis and serves as a marker for effective therapy with TCH,

either alone or in combination with chemotherapy. Results from randomized controlled studies show that adding TCH to first-line chemotherapy seems to be beneficial in women with metastatic breast cancer that overexpresses HER2.

The molecular characterization of childhood leukemias directly affects treatment strategies. ALL patients whose leukemic lymphoblasts contain the MLL–AF4 or the BCR–ABL fusion are often candidates for allo-HSCT during first remission. Patients with acute promyelocytic leukemia (PML) who carry the PML–RAR alpha fusion respond to all-trans-retinoic acid and have an excellent outcome after treatment with all-trans-retinoic acid in combination with anthracyclines.

16.16.3 Testing Microsatellite Instability for Response to Chemotherapy

Microsatellites are stretches of DNA in which a short motif (usually one to five nucleotides long) is repeated several times. Microsatellite instability (MSI) is considered to occur when a germ line microsatellite allele has gained or lost repeat units and has thus undergone a somatic change in length. Because this type of alteration can be detected only if many cells are affected by the same change, it is an indicator of the clonal expansion, which is typical of a neoplasm.

To test for MSI, DNA from the tumor and from normal tissue (blood, a buccal smear, or normal colonic mucosa) is tested by genotyping fluorescently labeled PCR products with the use of an automated sequencer. A panel of five microsatellite markers is usually adequate with MSI if two or more of them indicate a positive result. Such tests could help physicians determine a patient's prognosis and serve as a guide to therapy.

Fluorouracil-based adjuvant chemotherapy benefits patients with stage II or stage III colon cancer with microsatellite-stable tumors or tumors exhibiting low-frequency MSI but not those with tumors exhibiting high-frequency MSI. Although the results in vitro studies suggest that fluorouracil-based adjuvant chemotherapy is not beneficial in patients with colon cancer exhibiting high-frequency MSI, other drugs, such as the topoisomerase-I (TOP1) inhibitor camptothecin, have been shown to kill mismatch repair-deficient cancer cells exhibiting high-frequency MSI. Therefore, it would be important to conduct molecular analyses of specimens from recent clinical trials of non-fluorouracil-based chemotherapies and to ensure that future trials include analyses of molecular pathways. In this retrospective analysis, the finding that fluorouracil-based adjuvant chemotherapy does not significantly increase, and may potentially decrease, overall and disease-free survival among patients with tumors exhibiting high-frequency MSI raises several provocative issues regarding postoperative management of stage II and stage III colon cancer. Currently available evidence is not strong enough for decision-making in clinical practice. However, these findings, if confirmed by other analyses of previous, well-designed clinical trials or by future prospective, randomized, controlled studies, indicate that MSI testing should be conducted routinely and the results used to direct rational adjuvant chemotherapy in colon cancer.

16.17 Pharmacogenetics of Cancer Chemotherapy

Present clinical algorithms assign adjuvant chemotherapy according to prognosis, but clinical decision-making would be greatly improved if reliable predictive markers were available to identify which subsets of patients benefit most from treatment. Another problem is that unpredictable efficacy and high levels of systemic toxicity are common in cancer chemotherapy. Genetic variability in drug-metabolizing enzymes and signaling pathways affects chemotherapy-related toxicity and treatment outcome in cancer. Pharmacogenetics, therefore, is particularly appealing for oncology. Cytotoxicity to chemotherapy agents, 5-fluorouracil and docetaxel, which have distinct mechanisms of action, is a heritable trait varying with dose. Polymorphisms in thymidylate synthase (TS), MTHFR, and FCGR3A, as well as the polymorphic DNA repair genes XPD and XRCC1, influence response to chemotherapy and survival outcomes.

In breast cancer and CRC, polymorphisms in metabolic enzymes involved in tamoxifen and irinotecan therapies have led the FDA to address genetic factors relevant to patient consideration of treatment with these compounds. Tamoxifen therapeutic failure in breast cancer has been associated with reduced CYP2D6 activity due to inefficient activation of tamoxifen. Irinotecan toxicity in CRC is more common in patients with reduced-activity UGT1A alleles, resulting in excessive exposure to the potent SN-38 metabolite. In CRC and lung cancers, somatic mutations in the EGFR and downstream signaling molecules have been associated with the therapeutic outcome of EGFR-directed therapies. Current advances in single gene—UGT1A1, CYP2D6, EGFR, and K-ras—or multigene analysis contribute to optimizing breast, colorectal, and lung cancer therapy, highlighting how pharmacogenetics has helped in personalized decision-making for patient management (Snozek et al. 2009).

16.17.1 CYP1A2

The enzyme product of CYP1A2 is involved in a number of environmental carcinogens as well as anticancer drugs such as tamoxifen and drugs used for preventing nausea associated with chemotherapy such as ondansetron. Other therapeutic drugs metabolized by CYP1A2 include acetaminophen, amitriptyline, clomipramine, clozapine, diazepam, methadone, propranolol, and tacrine. This shows the complexity of situations that can be encountered with coadministration of drugs in cancer patients in the presence of carcinogens. There are marked interindividual differences in capacity for CYP1A2 induction, which correlate with genetic polymorphisms termed CYP1A2F. The identification of individuals who have different capacities for induction of CYP1A2 may be an indicator of increased risk of drug interactions or drug toxicity when treated with drugs metabolized by CYP1A2. Genotyping of cancer patients prior to treatment may help to individualize treatment to avoid adverse reactions and increase the effectiveness of therapy.

16.17.2 Thiopurine Methyltransferase

Polymorphisms in the thiopurine methyltransferase (TPMT) gene have been convincingly associated with the therapeutic efficacy and toxicity of thiopurine chemotherapeutic agents: 6-mercaptopurine and 6-thioguanine. TPMT-deficient patients are at high risk of developing severe hematopoietic toxicity if treated with conventional doses of thiopurines. Insights gained from studies of the TPMT polymorphism illustrate the potential of pharmacogenomics to optimize cancer therapy by avoiding toxic side effects in genetically distinct subgroups of patients.

Genetic polymorphism at this gene locus is associated with difficulty in achieving an effective dose of chemotherapeutic drugs in children with leukemia. Children with inherited TPMT deficiency exhibit severe hematopoietic toxicity when exposed to drugs such as 6-mercaptopurine, whereas those with a high activity form of the enzyme require high doses of the drug to achieve any clinical benefit. The TPMT polymorphism is relatively rare, with only about 1 % of the white population being homozygous for it, but, since these individuals show exaggerated toxic responses to normal doses of thiopurine, TPMT phenotype may be an important factor in the successful treatment of childhood leukemia. About 10 % of children with leukemia are intolerant to 6-mercaptopurine because of genetic defects in mercaptopurine inactivation by TPMT. Some centers already provide a diagnostic phenotyping service to guide the clinical use of 6-mercaptopurine.

A pharmacogenomic test, developed at St. Jude Children's Research Hospital, enables physicians to predetermine patients' TPMT activity levels based on whether or not they have inherited the alleles associated with TPMT deficiency. The test classifies patients according to normal, intermediate, and deficient levels of TPMT activity. Concordance between genotype and phenotype approaches 100 %. Patients classified as normal in activity—about 90 % of whites and blacks—are treated with conventional doses. Lower doses are tailored to avoid toxicity in deficient and intermediate patients, who represent about 10 % of each of these populations. The TPMT genetic test is well recognized in the effective clinical management of patients with ALL. Adjusting the dose of 6-mercaptopurine by a 10- to 15-fold decrease compared with conventional doses makes thiopurine as tolerable and effective in TPMT-deficient patients as it is in patients with normal activity levels.

16.17.3 Dihydropyrimidine Dehydrogenase

DPD is responsible for 80 % of the degradation of 5-fluorouracil (5-FU), a commonly used anticancer therapy. 5-FU is a prodrug that requires activation to 5-fluoro-2-deoxyuridine monophosphate (5-FdUMP) to exert antitumor activity. 5-FdUMP inhibits tumor cell replication via inhibition of thymidine synthase, an enzyme that is required for the synthesis of pyrimidine, and this inhibition slows down the tumor growth. Intravenously administered 5-FU is inactivated by DPD, an

enzyme that exhibits wide variations among individuals. Patients with low DPD accumulate excessive 5-FdUMP, which causes severe gastrointestinal and neurological toxicities.

Approximately 3 % of Caucasians have a deficiency of the enzyme DPD. Patients with a DPD deficiency who receive 5-FU have a prolonged half-life of the active compound and may experience life-threatening and even fatal toxicities including neurotoxicity and hematopoietic toxicity. On the other hand, overexpression of DPD in tumor tissues is associated with 5-fluorouracil resistance, as determined by GEP. This suggests the need to individualize therapy to avoid enhanced toxicity. Cimetidine is an inhibitor of DPD and, therefore, concomitant use of cimetidine with 5-FU can result in similar toxicities. There are numerous mutations that may occur, making the assay difficult to perform and standardize.

16.17.4 UGT1A1 Test as Guide to Irinotecan Therapy

Although most patients tolerate the chemotherapeutic agent irinotecan (Campostar®) for CRC quite well, some patients are genetically predisposed to severe side effects. Earlier studies with the irinotecan demonstrated that the highly variable toxicity was related to variability in the drug's metabolism. It was subsequently found that patients with two copies of one version of the UGT1A1 gene had few side effects at the standard dosage. Patients with only one copy of this version had more difficulty, and patients with two copies of the alternative version were at high risk for severe side effects. Therefore, relying on one standard dose meant that some of those patients received subtherapeutic doses of irinotecan and others received more than they could manage. UGT1A1 test was developed as a companion diagnostic to irinotecan therapy. The UGT1A1 test enables the physician to know in advance which patients are at risk. Those patients could be given reduced doses of irinotecan or other chemotherapy drugs. Genotyping results of UGT1A1 gene appear to predict severe adverse reactions more straightforward than the pharmacokinetic parameters or the phenotypes of the enzymatic activity.

16.18 Role of Computational Models in Personalized Anticancer Therapy

16.18.1 A Computational Model of Kinetically Tailored Treatment

Histological characteristics of a tumor are not a reliable indicator of the natural history. A mechanism-based framework using complementary DNA (cDNA) arrays and computational models has promise in improving diagnosis and prediction,

thereby making tailored therapy possible. Computational models may become important tools to help optimize and tailor cancer treatments. Ideal characteristics of anticancer drug development suitable for personalized approach are:

- Designed to inhibit specific biologic pathways involved in oncogenesis
- Mechanistic specificity rather than organ/tissue selectivity
- Should fit with initiatives in individualized therapy: cDNA arrays and computational models
- Synergistic with other chemotherapeutic agents
- Prevent or delay the emergence of resistance
- Transform cancer into a chronic disease by delaying time to progression

16.18.2 Mathematical Modeling of Tumor Microenvironments

The environment of a tumor is a crucial determining factor in its development. A multiscale mathematical model of cancer invasion, which considers cellular and microenvironmental factors simultaneously and interactively, can forecast how tumors grow and invade tissue (Anderson et al. 2006). The model simulations predict that harsh tumor microenvironment conditions (e.g., hypoxia, heterogeneous extracellular matrix) exert a dramatic selective force on the tumor, which grows as an invasive mass with fingering margins, dominated by a few clones with aggressive traits. In contrast, mild microenvironment conditions (e.g., normoxia, homogeneous matrix) allow clones with similar aggressive traits to coexist with less aggressive phenotypes in a heterogeneous tumor mass with smooth, noninvasive margins. Thus, the genetic makeup of a cancer cell may realize its invasive potential through a clonal evolution process driven by definable microenvironmental selective forces. The model shows a clear relationship between the shape of a cancer tumor and how aggressive it is. Aggressive tumors tend to assume a spidery shape in the model, while more benign growths are generally more spherical in shape. The findings would influence decision on how certain cancers are treated, by considering the environment around the tumor to be a contributory factor in how aggressive the cancer. Most of the current treatments are focused on making the tissue environment as harsh as possible for the tumor in the hope of destroying it. But this could allow the most aggressive cancer cells to dominate any residual tumor left after treatment and develop resistance to treatment. Moreover, these aggressive cells tend to be the more invasive resulting in an increased chance of metastasis. With the use of the tools of mathematical modeling and computer simulation, cancer treatment will no longer be a trial-and-error game. With mathematics-driven oncology research, it will be possible to determine which drugs will work at which stage. In the future this research could help personalize treatment in a patient-specific manner.

16.19 Therapy Resistance in Cancer

Human cancers are mostly found to be resistant to therapy at the time of drug presentation (primary responses), tumors being intrinsically drug resistant (innate or de novo drug resistance). Only a few become resistant after an initial response (acquired responses), the tumors developing resistance to chemotherapy during treatment (acquired drug resistance). In the latter group, a tumor cell may express drug resistance by combining several distinct mechanisms induced by its exposure to various drugs. In the former group, however, this is unlikely to be the case.

16.19.1 Mechanism of Therapy Resistance in Cancer

One explanation of development of resistance is that when cells become cancerous, they also become 100 times more likely to genetically mutate than regular cells. Mutations protect cancer cells from therapeutics designed to target a particular oncogene. A single tumor may have cells with many different types of oncogenes and drug-resistant genes. Molecular diagnostics will help determine the stage and malignancy of a tumor by testing the number of its mutations. The more mutations, the further along the tumor may be in its development to malignancy or metastasis. Resistance to drugs that shut down oncoprotein-driven pathways can occur because of compensatory changes in connecting pathways. The loss of expression of MED12, which acts in the TGF-β signaling pathway, may mediate resistance to gefitinib and vemurafenib (Rosell 2013).

The mechanism underlying multidrug resistance (MDR) is a cellular pump called P-glycoprotein, which normally protects cells from toxic substances by actively exporting the offending compounds.

Pharmacogenetics and pharmacogenomics studies of the relationship between individual variations and drug response rates reveal that genetic polymorphisms of specific genes is associated with clinical outcomes in patients treated through chemotherapy, and amplification of genes encoding drug targets or transporters alters the sensitivity of cancer cells to a particular chemotherapy. The LOH at specific regions of chromosomes has been identified in specific cancers but its effect on treatment outcome remains controversial.

16.19.1.1 Overexpression of Multidrug Resistance Gene

Approximately 75 % of cancer patients are intrinsically unresponsive or develop resistance to anticancer drugs. The mechanism underlying MDR is a cellular pump called P-glycoprotein. Under normal circumstances, P-glycoprotein protects cells from toxic substances by actively exporting the offending compounds. In cancer,

abundant P-glycoprotein gene (MDR-1) expression by a tumor has been implicated as one of the major reasons that cancer cells develop resistance to chemotherapy. Overexpression of MDR-1 in tumors has been associated with resistance to Adriamycin, paclitaxel, and many more anticancer drugs. A simple DNA test has been devised by Epidauros Biotechnology that enables a physician to predict drug uptake from the beginning of therapy of cancer and avoid the trial-and-error approach. This test for the detection of gene polymorphisms is based on the knowledge that MDR-1 has 15 polymorphisms of which only one correlates with poor drug uptake. Once detected, management of drug resistance is still problematic as there is no ideal remedy for it. One compound, OC 144-093 (Ontogen Corporation, Carlsbad, CA), has passed phase I single-blind, placebo-controlled trials. This compound is orally active, nontoxic and does not interact with paclitaxel.

16.19.1.2 P53 Mutations

The function of the human p53 gene, sometimes associated with drug resistance, remains only partially understood. In response to cellular stresses such as DNA damage or oncogene activation, p53 acts as a tumor suppressor by blocking cell division or inducing cell suicide through apoptosis. If p53 is mutated or otherwise inactivated, a cell can accumulate further mutations that lead to tumor formation. Furthermore, tumor cells with mutant p53 are typically unable to invoke apoptosis in response to DNA damage, rendering such tumors resistant to traditional chemotherapy and radiation therapy.

16.19.2 Detection of Drug Resistance

16.19.2.1 Anaplastic Lymphoma Kinase

Anaplastic lymphoma kinase (ALK) is a RTK of the insulin receptor superfamily. Translocations (fusions) of ALK have an established pathogenic role in more than 250,000 new cancer diagnoses in the USA each year. Detection of ALK mutations has been considered increasingly important in the diagnosis and therapy selection for many types of cancer, including NSCLC, diffuse large-B-cell lymphoma, anaplastic large-cell lymphoma, neuroblastoma, and inflammatory myofibroblastic tumors. Because of the potential for ALK-inhibitor therapies to treat so many cancers, there are several ALK inhibitors currently in development by pharmaceutical firms. ALK assays (Insight Genetics) are based on the need for better methods of not only detecting activating ALK fusions and upregulation across many cancer types but also monitoring for resistance mutations that arise in response to ALK-inhibitor therapy. Insight ALK Screen assay provides quick, accurate detection of

any ALK fusion. Insight ALK Resistance Monitoring assays assist in monitoring patients for ALK resistance mutations.

16.19.2.2 Metabolic Profiling of Cancer

Acquired resistance to imatinib mesylate is an increasing and continued challenge in the treatment of BCR–ABL tyrosine kinase-positive leukemias as well as GISTs. Stable isotope-based dynamic metabolic profiling (SIDMAP) studies conducted in parallel with the development and clinical testing of imatinib have revealed that this targeted drug is most effective in controlling glucose transport, direct glucose oxidation for RNA ribose synthesis in the pentose cycle, as well as de novo long-chain fatty acid synthesis. Thus imatinib deprives transformed cells of the key substrate of macromolecule synthesis, malignant cell proliferation, and growth. Tracer-based MRS studies revealed a restitution of mitochondrial glucose metabolism and an increased energy state by reversing the Warburg effect, consistent with a subsequent decrease in anaerobic glycolysis. In vitro SIDMAP studies that involved myeloid cells isolated from patients who developed resistance against imatinib have indicated that nonoxidative ribose synthesis from glucose and decreased mitochondrial glucose oxidation are reliable metabolic signatures of drug resistance and disease progression. There is also evidence that imatinib-resistant cells utilize alternate substrates for macromolecule synthesis to overcome limited glucose transport controlled by imatinib. The main clinical implications involve early detection of imatinib resistance and the identification of new metabolic enzyme targets with the potential of overcoming drug resistance downstream of the various genetic and BCR–ABL expression-derived mechanisms. Metabolic profiling is an essential tool used to predict, clinically detect, and treat targeted drug resistance. This need arises from the fact that targeted drugs are narrowly conceived against genes and proteins but the metabolic network is inherently complex and flexible to activate alternative macromolecule synthesis pathways that targeted drugs fail to control.

16.19.3 Management of Drug Resistance in Cancer

16.19.3.1 Chemogenomic Approach to Drug Resistance

Resistance to anticancer drugs represents a serious obstacle to successful cancer treatment. Genome-wide studies correlating drug response phenotypes with large DNA/tissue microarray and proteomic datasets have been performed to identify the genes and proteins involved in chemosensitivity or drug resistance. The goal is to identify a set of chemosensitivity and/or resistance genes for each drug that are predictive of treatment response. Therefore, validated pharmacogenomic

biomarkers offer the potential for the selection of optimal treatment regimens for individual patients and for identifying novel therapeutic targets to overcome drug resistance.

Approximately 10 % of patients with chemotherapy-resistant bowel cancer that has spread to other parts of the body respond to treatment with MAbs—cetuximab or panitumumab. These drugs target the EGFR. Understanding the molecular basis of clinical sensitivity or resistance to anti-EGFR agents might identify patients who are likely to benefit from treatment with these MAbs. Patients that are responsive to anti-EGFR antibody treatment have an increased number of copies of the EGFR gene when compared with patients that do not respond to treatment suggesting that MAbs are likely to be more effective against gene targets in cancer that are amplified rather than those affected by point mutations. Therefore, assessment of EGFR copy number might identify patients with metastatic CRC who are likely to respond to MAbs against EGFR. Those not likely to respond would be spared the expense and potential adverse effects of this treatment.

16.19.3.2 Determination of Chemotherapy Response by Topoisomerase Levels

Topoisomerase poisons are chemotherapeutic agents that are used extensively for treating human malignancies. These drugs can be highly effective, yet tumors are frequently refractory to treatment or become resistant upon tumor relapse. Top2A expression levels are major determinants of response to the topoisomerase-2 poison doxorubicin and suppression of Top2A produces resistance to doxorubicin. Suppression of Top1 produces resistance to the topoisomerase 1 poison camptothecin but hypersensitizes cancer cells to doxorubicin. Lymphomas relapsing after treatment display spontaneous changes in topoisomerase levels as predicted by in vitro gene knockdown studies using RNAi screens in animal models of cancer. Thus pooled shRNA screens can be used for identifying genetic determinants (biomarkers) of chemotherapy response and improve the effectiveness of topoisomerase poisons in the clinic (Burgess et al. 2008).

16.19.3.3 Management of Drug Resistance in Leukemia

Imatinib mesylate (Novartis' Gleevec) causes remission in patients with CML. Despite these positive response rates, a subset of patients do not respond to Gleevec therapy fully or at all, and approximately 4–5 % of successfully treated patients annually develop resistance to Gleevec during therapy with a return of their disease manifestations. The molecular hallmark of CML is a mutation known as BCR–ABL. This mutation is the specific target for Gleevec and is found in 95 % of patients with CML. Secondary mutations in the ABL portion of the gene correlate with treatment failure or relapse in most patients on Gleevec therapy.

A novel pyrido[2,3-d]pyrimidine derivative, PD180970, has been shown to potently inhibit Bcr–Abl and induce apoptosis in Bcr–Abl-expressing leukemic cells in patients who develop a resistance to Gleevec. Developing additional Abl kinase inhibitors would be useful as a treatment strategy for chronic myelogenous leukemia. The key to curing more CML patients is to provide customized treatment for each individual, based on the particular molecular mutation that causes their resistance to Gleevec. Leukemia cells from patients with advanced CML should be profiled and the appropriate inhibitor or combination of inhibitors selected for treatment. This approach is similar to the method that has been used to treat HIV drug resistance. Treatment would be individualized for each patient, by combining specific inhibitors in an "inhibitor cocktail" that would be able to combat various Bcr–Abl isoforms. The paradigm is to understand the genetic abnormality that drives the growth and survival of cancer and tailor a treatment to reverse this genetic defect.

16.19.3.4 Resistance to Vaccines in Cancer Recurrence After Surgery

Of the >700,000 patients who undergo cancer surgery in the USA each year, >40 % develop recurrences that have a poor outcome. Recurrent tumor cells have few phenotypical differences from those in tumors prior to surgery. An alternative explanation proposed for the resistance of recurrent tumors is that surgery promotes inhibitory factors that allow lingering immunosuppressive cells to repopulate small pockets of residual disease quickly (Predina et al. 2013). These authors found that recurrent tumors and draining lymph nodes are infiltrated with M2 macrophages and $CD4^+Foxp3^+$ regulatory T cells. This complex network of immunosuppression in the tumor microenvironment explains the resistance of tumor recurrences to conventional cancer vaccines despite small tumor size, an intact antitumor immune response, and unaltered cancer cells. Therapeutic strategies coupling anticancer agents with inhibition of immunosuppressive cells potentially could impact the outcomes in these patients.

16.19.3.5 Systems Biology Approach to Drug-Resistant Cancer

Resistance to targeted cancer therapies such as TCH may occur not only because of insufficient expression of HER2 receptor but also because of the overriding activation states of cell signaling pathways. Systems biology approaches lend themselves to rapid in silico testing of factors, which may confer resistance to targeted therapies. A new kinetic model could be interrogated to predict resistance to RTK inhibitor therapies and directly test predictions in vitro and in clinical samples (Faratian et al. 2009). The mathematical model includes RTK inhibitor antibody binding, HER2/HER3 dimerization and inhibition, AKT/mitogen-activated protein kinase cross talk, and the regulatory properties of PTEN. The model includes parameters based on quantitative phosphoprotein expression data from cancer cell lines using

reverse-phase protein microarrays. Quantitative PTEN protein expression was found to be the key determinant of resistance to anti-HER2 therapy in silico, which was predictive of virtual experiments in vitro using the PTEN inhibitor bp(V). When measured in cancer cell lines, PTEN expression predicts sensitivity to anti-HER2 therapy; furthermore, this quantitative measurement is more predictive of response than other pathway components taken in isolation and when tested by multivariate analysis in a cohort of 122 breast cancers treated with TCH. Thus a systems biology approach has been successfully used to stratify patients for personalized therapy in cancer and is further compelling evidence that PTEN, appropriately measured in the clinical setting, refines clinical decision-making in patients treated with anti-HER2 therapies.

16.20 Personalized Therapy of Cancer Metastases

Metastasis is the major cause of mortality in cancer. Early detection of metastases is important.

MetaStat Inc. has identified a dangerous isoform of mena, Mena^{INV} (mena invasive), which is not present in normal cells in the adults. Mena^{INV} potentiates EGF-induced membrane protrusion and increases the matrix degradation activity of tumor cells (Philippar et al. 2008). Mena^{INV} is a target for potential therapy for cancer metastases.

16.21 Personalized Management of Cancers of Various Organs

16.21.1 Personalized Management of Brain Tumors

Brain tumors can be benign or malignant, with the latter being more frequent. Most of the discussion in this section is about GBM, which is the most malignant and most frequent brain tumor and is currently incurable with a median survival of <2 years after diagnosis and treatment. Worldwide ~175,000 cases occur annually of which 17,000 are diagnosed in the USA. Several innovative treatments are being developed, but the mainstays of conventional treatment are chemotherapy and radiation. Chemotherapy gives inconsistent results in terms of prolongation of survival. GBM is a complex, heterogeneous disease, which makes it unlikely that a uniform approach would be suitable for all patients. There is a need for the development of personalized treatment modalities to address the heterogeneity of this complex tumor phenotype.

16.21.1.1 Aptamers for Selective Targeting of Tumor-Initiating Cells in GBM

GBM displays cellular hierarchy with self-renewing tumor-initiating cells (TICs), also known as CSCs, at the apex. Although the TIC hypothesis remains controversial and the functional assays to define the TIC phenotype are evolving, it has been shown that TICs may contribute to angiogenesis, spread of tumor, and resistance to therapy. However, the identification of TICs by the use of biomarkers characterized in normal stem cells has an inherent limitation to selectively identify TICs. A study adopted Cell-Systematic Evolution of Ligands by Exponential Enrichment (Cell-SELEX) to identify aptamers that specifically bind to TICs in GBM but not to human neural stem cells (Kim et al. 2013). These aptamers select and internalize into GBM cells that self-renew, proliferate, and initiate tumors. As aptamers can be modified to deliver payloads, aptamers may represent novel agents that could selectively target or facilitate imaging of TICs, which may be important for improving therapeutic outcomes in individual patients.

16.21.1.2 Biosimulation Approach to Personalizing Treatment of Brain Cancer

Gene Network Sciences (GNS), using its REFS™ (Reverse Engineering and Forward Simulation) technology, is collaborating with MD Anderson Cancer Center (Houston, TX) to translate DNA sequence and clinical data from GBM patients into breakthrough discoveries leading to drugs and diagnostics. The results from these projects will include the identification of new combination drug targets for disease and the development of diagnostics to determine appropriate individual patient treatments. The parties plan to transform this coherent clinical 3D data into computer models which link genetic alterations to changes in gene expression to progression-free patient survival times. This computer model, developed by using the REFS™ platform, is expected to unravel the complex genetic circuitry underlying GBM and reveal novel drug targets and biomarkers of response. These targets and biomarkers may be used to identify the optimal single or combination drug therapy for a given patient's genetic alteration profile. The parties will utilize MD Anderson's clinical expertise to validate the discoveries and will work with strategic partners to make drugs and diagnostics stemming from these discoveries available to patients.

16.21.1.3 Companion Diagnostic for Viral Gene Therapy of Brain Cancer

Toca 511 (vocimagene amiretrorepvec), an injectable, and Toca FC (flucytosine), an extended-release tablet, are formulations of a retroviral replicating vector for delivering a cytosine deaminase gene selectively to cancer cells. After Toca 511 spreads through a tumor, the cancer cells expressing the cytosine deaminase gene may convert the antibiotic flucytosine into the anticancer drug 5-fluorouracil. Tocagen, the

manufacturer of Toca 511 and Toca FC, has given Siemens Healthcare Diagnostics commercialization rights to diagnostic tests for monitoring the levels of viral gene therapy for brain cancer. Tocagen is enrolling patients for its clinical trials and will partner with Siemens on assays used for the trials.

16.21.1.4 Drug Resistance in GBM

Despite their nearly universal activation of mTOR signaling, GBMs are strikingly resistant to mTOR-targeted therapy. Analysis of GBM cell lines, patient-derived tumor cell cultures, and clinical samples from patients in phase I clinical trials has revealed that the PML gene mediates resistance to mTOR-targeted therapies (Iwanami et al. 2013). Direct mTOR inhibitors and EGFR inhibitors that block downstream mTOR signaling promote nuclear PML expression in GBMs. Genetic overexpression and knockdown approaches demonstrate that PML prevents mTOR and EGFR inhibitor-dependent cell death. Low doses of the PML inhibitor, arsenic trioxide, abrogate PML expression and reverse mTOR kinase inhibitor resistance in vivo, thus markedly inhibiting tumor growth and promoting tumor cell death in mice. These results identify a unique role for PML in mTOR and EGFR inhibitor resistance and provide a strong rationale for a combination therapeutic strategy to overcome it.

Intratumor heterogeneity of GBM is likely the key to understanding treatment failure or drug resistance. An integrated genomic analysis of spatially distinct tumor fragments has been developed to uncover extensive intratumor heterogeneity (Sottoriva et al. 2013). Phylogeny of the fragments for each patient was reconstructed by identifying copy number alterations in EGFR and CDKN2A/B/p14ARF as early events and aberrations in PDGFRA and PTEN as later events during cancer progression. Results of the study revealed patient-specific patterns of cancer evolution to enable more effective personalized treatment design.

16.21.1.5 Genetics and Genomics of Brain Cancer

Genetic alterations in GBM have been studied extensively using molecular diagnostic technologies. GEP reveals extensive differences in gene expression among GBMs, particularly in genes involved in angiogenesis, immune cell infiltration, and extracellular matrix remodeling. One gene, FABP7, is associated with survival and is a prognostic marker of both biological and clinical significance. A team of scientists from the Institut Curie and Inserm in France have harnessed the technology of DNA chips to identify tumors with the best prognosis, whose chromosome 1 has undergone a specific deletion (Idbaih et al. 2005). Several types of deletions of chromosome 1 have been identified but only the complete loss of the short arm of chromosome 1 combined with complete loss of the long arm of chromosome 19 signifies a good prognosis. Partial loss of the short arm of chromosome 1, on the other hand, characterized more aggressive tumors. Results were obtained by studying the specific genetic alterations of a subgroup of more chemosensitive gliomas.

These findings were recorded using high-density array comparative genomic hybridization (CGH) analysis. CGH chips are made by using targets from genome fragments of about 150,000 base pairs. With some 3,500 targets, these chips afford an overview of the whole genome. This technique that can establish high-resolution maps reveals genome anomalies (amplifications, deletions). Screening for these deletions can be incorporated into standard diagnostic tests for GBM. In using these tools, physicians can revamp and refine tumor classification to enable more individualized treatment. Expression profiling combined with mutation analysis has an important role in the development of rational therapies for GBM.

Genetic differences may also have indirect effects on drug response that are unrelated to drug metabolism or transport, such as methylation of the methylguanine methyltransferase (MGMT) gene promoter, which alters the response of glioblastoma (malignant brain tumor) to treatment with carmustine. The mechanism of this effect is related to a decrease in the efficiency of repair of alkylated DNA in patients with methylated MGMT.

Activation of the transcription factor STAT3 is considered to potently promote oncogenesis in a variety of tumors including GBM leading to intense efforts to develop STAT3 inhibitors for treatment. However, the function of STAT3 in GBM pathogenesis has remained unknown. STAT3 is a key gene that turns neural stem cells into astrocytes during normal development. One study reports that STAT3 plays a pro-oncogenic or tumor-suppressive role depending on the mutational profile of the tumor (de la Iglesia et al. 2008). Deficiency of the tumor suppressor PTEN triggers a cascade that inhibits STAT3 signaling in murine astrocytes and human GBM. Specifically, there is a direct link between the PTEN–Akt–FOXO axis and the leukemia inhibitory factor receptor β (LIFRβ)–STAT3 signaling pathway. Accordingly, PTEN knockdown induces efficient malignant transformation of astrocytes upon knockout of the STAT3 gene. Remarkably, in contrast to the tumor-suppressive function of STAT3 in the PTEN pathway, STAT3 forms a complex with the oncoprotein EGFR type III variant (EGFRvIII) in the nucleus and thereby mediates EGFRvIII-induced glial transformation. In short, when EGFR is mutated, STAT3 is an oncogene; with a PTEN mutation, STAT3 is a tumor suppressor. These findings indicate that STAT3 plays opposing roles in glial transformation depending on the genetic background of the tumor, providing the rationale for personalized treatment of GBM. STAT3 has also been implicated in prostate and breast cancers, so these results may translate to other types of tumors as well.

Mutations of EGFR are found in over 50 % of GBMs. Concomitant activation of wild-type and/or mutant (vIII) EGFR and ablation of Ink4A/Arf and PTEN tumor suppressor gene function in the adult mouse CNS induces rapid onset of an infiltrating, high-grade malignant glioma phenotype with prominent pathological and molecular resemblance to GBM in humans (Zhu et al. 2009). Studies of the activation of signaling events in these GBM tumor cells revealed notable differences between wild-type and vIII EGFR-expressing cells. Whereas wild-type EGF receptor signals through its canonical pathways, tumors arising from expression of mutant EGFRvIII do not use these same pathways. These findings provide critical insights into the role of mutant EGFR signaling function in GBM tumor biology and set the stage for testing of targeted therapeutic agents in suitable preclinical models.

A comprehensive analysis using NGS technologies has led to the discovery of a variety of genes that were not known to be altered in GBMs (Parsons et al. 2008). There were recurrent mutations in the active site of isocitrate dehydrogenase 1 (IDH1) in 12 % of GBM patients; these occurred in a large fraction of young patients and in most patients with secondary GBMs and were associated with an increase in overall survival. These studies demonstrate the value of unbiased genomic analyses in the characterization of human brain cancer and identify a potentially useful genetic alteration for the classification and targeted therapy of GBMs.

Nuclear factor-kappaB (NF-κB) activation may play an important role in the pathogenesis of cancer and also in resistance to treatment. Inactivation of the p53 tumor suppressor is a key component of the multistep evolution of most cancers. Links between the NF-κB and p53 pathways are under intense investigation. Receptor-interacting protein 1 (RIP1), a central component of the NF-κB signaling network, negatively regulates p53 tumor suppressor signaling (Park et al. 2009b). The loss of RIP1 from cells results in augmented induction of p53 in response to DNA damage, whereas increased RIP1 level leads to a complete shutdown of DNA damage-induced p53 induction by enhancing levels of cellular mdm2. The key signal generated by RIP1 to upregulate mdm2 and inhibit p53 is the activation of NF-κB. The clinical implication of this finding is shown in GBM, where RIP1 is commonly overexpressed, but not in grade II and III glioma. RIP1 activates NF-κB and then that increases the expression of the gene mdm2, which inhibits the p53 gene in GBM. Increased expression of RIP1 confers a worse prognosis. These results show a key interaction between the NF-κB and p53 pathways that may have implications for the targeted treatment of glioblastoma. One of the next steps is to determine whether these patients may respond better to drugs targeting the NF-κB network.

16.21.1.6 Glioma Actively Personalized Vaccine Consortium

On 5 July 2013, a research consortium, Glioma Actively Personalized Vaccine Consortium (GAPVAC) consisting of 14 partners from seven European countries plus the USA, led by Immatics Biotechnologies announced the plans to bring a personalized brain tumor therapy into clinical phase I. The EU has supported the endeavor to adopt a personalized medicine approach with €6m through its 7th Framework Program. The GAPVAC project is designed to create, manufacture, and develop actively personalized vaccines (APVACs) tailored for each patient based on the individual aspects of the patient's tumor and immune system. The latest technologies, including NGS, high-sensitivity MS, and innovative immunomonitoring approaches, will be combined to generate an optimal therapy for the individual patient. At the core of the project is a phase I clinical trial which will enroll up to 30 newly diagnosed glioblastoma patients and is expected to start in 2014. Glioblastoma patients will be repetitively immunized with a vaccine specifically prepared for them. The clinical trial will be accompanied by an extensive biomarker program to confirm the mechanism of action and to identify biomarker signature candidates predicting which patients are most likely to benefit from treatment with APVACs.

16.21.1.7 Prognosis of Glioblastoma Multiforme Based on Its Genetic Landscape

The alteration of multiple networking genes by recurrent chromosomal aberrations in gliomas deregulates critical signaling pathways through multiple, cooperative mechanisms (Bredel et al. 2009). These mutations, which are likely due to nonrandom selection of a distinct genetic landscape during gliomagenesis, are associated with patient prognosis.

A clinical study has shown that 14-3-3zeta-positive expression was observed in approximately 74.5 % of patients with GBM who had lower overall survival rates and median survival time than those in the 14-3-3zeta-negative group (Yang et al. 2011). 14-3-3Zeta-positive expression in tumor cells also was correlated with a shorter interval to tumor recurrence. Univariate and multivariate analyses showed that 14-3-3zeta-positive expression was an independent prognostic factor for GBM and can be used as a biomarker.

GBMs often have both monosomy of chromosome 10 and gains of the EGFR gene locus on chromosome 7. Chromosome 10 losses that decrease tumor suppressor gene ANXA7 levels correspond to a rise in EGFR levels that increase tumor aggressiveness and decrease survival times. This provides a clinically relevant mechanism to augment EGFR signaling in glioblastomas beyond that resulting from amplification of the EGFR gene (Yadav et al. 2009). Further work is continuing to characterize the mechanism by which ANXA7 regulates EGFR.

Seven of the 31 most intriguing landscape genes are independently associated with patient survival in GBM: POLD2, CYCS, myc, AKR1C3, YME1L1, ANXA7, and PDCD4. This seven-gene set could retrospectively classify patients into subgroups linked to survival times. Individuals who have alterations in between zero and two of the seven genes are classified as low risk, while those with five or more affected genes are considered high risk. Those in between are classified as high risk. This type of approach could have clinical applications for both improving brain tumor classification methods (currently based on histology and clinical factors such as age) and guiding treatment decisions. These findings will spur the development of new therapies based on key brain cancer pathways. Prospective clinical trials are planned for testing the clinical utility of the seven-gene set. A similar genetic landscape approach may be applied to other aggressive types of cancer, such as ovarian and lung cancer. Eventually, networks may be created that account for both genetic and epigenetic changes in cancer cells.

16.21.1.8 Molecular Diagnostics for Personalized Management of Brain Cancer

Several molecular biomarkers have been identified in diffuse gliomas that carry diagnostic and prognostic information. In addition, some of these and other biomarkers predict the response of these gliomas to particular chemotherapeutic approaches. Molecular diagnostics is an important contribution to personalized management of glioma patients.

Diffusion MRI as a biomarker. The response to treatment of brain cancer is usually assessed by measurements obtained from brain imaging several months after the start of treatment. A biomarker of tumor response would be useful for making early treatment decisions and for determining prognosis. To obtain this information, patients with malignant glioma are examined by diffusion MRI before treatment and 3 weeks after treatment; the images are coregistered, and differences in tumor-water diffusion values are calculated as functional diffusion maps (fDMs), which are correlated with the radiographic response, time to progression, and overall survival. Changes in fDM at 3 weeks are closely associated with the radiographic response at 10 weeks. The percentage of the tumor undergoing a significant change in the diffusion of water is different in patients with progressive disease as compared to those with stable disease. fDM provides an early biomarker for response, time to progression, and overall survival in patients with malignant glioma. This method has the potential to evaluate differences in efficacy between patients as well as to assess the heterogeneity of response within an individual tumor. This technique should be further evaluated to determine its usefulness in the individualization of treatment or evaluation of the response to treatment in clinical trials.

Combined neuroimaging and DNA microarray analysis. This method has been used to create a multidimensional map of gene expression patterns in GBM that provides clinically relevant insights into tumor biology (Diehn et al. 2008). Tumor contrast enhancement and mass effect can predict the activation of specific hypoxia and proliferation gene expression programs, respectively. Overexpression of EGFR, a RTK and potential therapeutic target, has also been directly inferred by neuroimaging and validated in an independent set of tumors by IHC. Furthermore, imaging provides insights into the intratumoral distribution of gene expression patterns within GBM. An "infiltrative" imaging phenotype can identify and predict patient outcome. Patients with this imaging phenotype have a greater tendency toward having multiple tumor foci and demonstrate significantly shorter survival than their counterparts. These findings provide an in vivo portrait of genome-wide gene expression in GBM and offer a potential strategy for noninvasively selecting patients who may be candidates for individualized therapies.

Proteomics of brain cancer. Protein biomarkers of brain tumors have potential clinical usefulness for predicting efficacy of anticancer agents. Surgical samples of human gliomas can be analyzed with two dimensional gel electrophoresis (2D GE) and MS and in vitro chemosensitivities to various anticancer agents can be measured by flow cytometric detection of apoptosis. Proteins that are significantly affected the in vitro chemosensitivity to each category of anticancer agents can be identified that may be the potential predictive markers for chemosensitivity in human gliomas.

Epigenetic biomarkers of GBM. One of the most intrigued subtypes is the long-term survival GBM, which responds better to current therapies. An investigation based on molecular epigenetic, clinical, and histopathological analyses was carried out to identify biomarkers useful for distinguishing long-term survival form from classic GBM (Martinez et al. 2007). It involved analysis of the promoter methylation status of key regulator genes implicated in tumor invasion (TIMP2, TIMP3),

apoptosis, and inflammation (TMS1/ASC, DAPK) as well as overall survival, therapy status, and tumor pathological features. A methylation-specific PCR approach was performed to analyze the CpG island promoter methylation status of each gene. The results of this study indicate that, compared to classic GBM, long-term survival form of GBM displays distinct epigenetic characteristics, which might provide additional prognostic biomarkers for the assessment of this malignancy.

O6-methylguanine methyltransferase (MGMT) promoter methylation has been observed in a considerable proportion of all grades and subtypes of gliomas, with no significant correlation with other known genetic alterations. On extensive literature review, in both low- and high-grade gliomas, a wide variability of data on the frequency of MGMT methylation and its association with other molecular alterations from various centers was noted, mostly owing to technical causes (Jha et al. 2010). This raises questions regarding the capacity of this test for use as an objective and reproducible biomarker for customized treatment in individual cases.

Multigene predictor of outcome in GBM. No single biomarker is a predictor of outcome in GBM. An analysis using GBM microarray data from four independent datasets of the genes consistently associated with patient outcome revealed a consensus 38-gene survival set (Colman et al. 2010). Worse outcome was associated with increased expression of genes associated with mesenchymal differentiation and angiogenesis. Application to formalin-fixed paraffin-embedded (FFPE) samples using real-time reverse transcriptase polymerase chain reaction (RT-PCR) assays resulted in a nine-gene subset which appeared robust in these samples. This nine-gene set was then validated in an additional independent sample set. Multivariate analysis confirmed that the nine-gene set was an independent predictor of outcome after adjusting for clinical factors and methylation of the methylguanine methyltransferase promoter. The nine-gene profile was also positively associated with biomarkers of glioma stem-like cells, including CD133 and nestin. Finally, a multigene predictor of outcome in GBM was identified, which is applicable to routinely processed FFPE samples. The profile has potential clinical application both for optimization of therapy in GBM and for the identification of novel therapies targeting tumors refractory to standard therapy. The assay is commercially available as DecisionDx-GBM (Castle Biosciences Inc.).

16.21.1.9 Personalized Chemotherapy of Brain Tumors

Although approximately 26 % of patients treated with temozolomide survive >2 years, it is difficult to predict who would respond to therapy. A number of tests are used to determine the responsiveness of GBM to chemotherapy.

Gene promoter methylation testing (MDxHealth Inc.). A clinical trial conducted at the University Hospital of Lausanne in Switzerland found that activity status of a single gene could predict response to therapy (Hegi et al. 2005). The O6-methylguanine-DNA methyltransferase (MGMT) promoter was methylated in 45 % of 206 assessable cases. Irrespective of treatment, MGMT promoter

methylation was an independent favorable prognostic factor. Among patients whose tumor contained a methylated MGMT promoter, a survival benefit was observed in patients treated with temozolomide and radiotherapy; their median survival was 21.7 months as compared with 15.3 months among those who were assigned to only radiotherapy. In the absence of methylation of the MGMT promoter, there was a smaller and statistically insignificant difference in survival between the treatment groups. Testing for the methylation status of the MGMT gene by PCR could lead to the use of temozolomide as first-line therapy in those identified as responder patients. Further analysis of the genetic pattern of the tumor after biopsy might provide new drug targets for the disease. Stratification according to MGMT promoter methylation status may be considered in the future trials in which temozolomide or other alkylating agents are used.

MDxHealth is conducting gene promoter methylation testing in a phase II clinical trial (CORE trial) for cilengitide (Merck KGaA) in newly diagnosed GBM patients. In addition, testing is also being performed in a phase III clinical trial (CENTRIC trial) in newly diagnosed glioblastoma that has been running since 2008. Patient selection for those trials is based on the MGMT gene promoter methylation status of their tumor tissue.

Molecular determinants of response to EGFR inhibitors. EGFR is amplified, overexpressed, or mutated in 50 % of GBM cases, but only 10–20 % of patients have a response to EGFR kinase inhibitors. In patients with recurrent GBM, coexpression of EGFRvIII and PTEN by tumor cells is associated with responsiveness to EGFR kinase inhibitors. One inherent resistance mechanism to EGFR inhibitors in GBM is the coactivation of multiple RTKs, which generates redundancy in the activation of phosphoinositide 3-kinase (PI3K) signaling. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) tumor suppressor is frequently phosphorylated at a conserved tyrosine residue, Y240, in GBM clinical samples (Fenton et al. 2012). Phosphorylation of Y240 is associated with shortened overall survival and resistance to EGFR inhibitor therapy in GBM patients and plays an active role in mediating resistance to EGFR inhibition in vitro. Y240 phosphorylation can be mediated by both fibroblast growth factor (FGF) receptors and SRC family kinases but does not affect the ability of PTEN to antagonize PI3K signaling. These findings show that, in addition to genetic loss and mutation of PTEN, its modulation by tyrosine phosphorylation has important implications for the development and treatment of GBM.

Simulating chemotherapeutic schemes for individualization. A novel patient individualized, spatiotemporal Monte Carlo simulation model of tumor response to chemotherapeutic schemes in vivo has been described (Stamatakos et al. 2006). Treatment of GBM by temozolomide is considered as a paradigm. The model is based on the patient's imaging, histopathologic and genetic data. A mesh is superimposed upon the anatomical region of interest, and within each geometrical cell of the mesh, the most prominent biological "laws" (cell cycling, apoptosis, etc.) in conjunction with pharmacokinetic and pharmacodynamic information are applied. A good qualitative agreement of the model's predictions with clinical experience supports the applicability of the approach to chemotherapy optimization.

Personalized therapy of GBM based on cancer stem cells (*CSCs*). CSCs play an important role in determining GBM response to therapy. Hypoxia and stem cell maintenance pathways may provide therapeutic targets to sensitize CSCs to cytotoxic therapies to improve GBM patient treatments. Although chemotherapy with temozolomide may contain tumor growth for some months, invariable GBM recurrence suggests that CSCs maintaining these tumors persist. According to a study of the effect of temozolomide on CSC lines, although differentiated tumor cells constituting the bulk of all tumor cells were resistant to the cytotoxic effects of the substance, temozolomide induced a dose- and time-dependent decline of the stem cell subpopulation (Beier et al. 2008). Temozolomide concentrations that are reached in patients are only sufficient to completely eliminate CSC in vitro from MGMT-negative but not from MGMT-positive tumors. These data strongly suggest that optimized temozolomide chemotherapeutic protocols based on MGMT status of CSCs might substantially improve the elimination of GBM stem cells and consequently prolong the survival of patients.

16.21.1.10 Supratentorial Hemispheric Diffuse Low-Grade Gliomas

Supratentorial hemispheric diffuse low-grade gliomas (LGG), i.e., World Health Organization (WHO) grade II gliomas, are a heterogeneous group of tumors. During their natural course, LGG tend to progress to a higher grade of malignancy, leading to neurological disability and ultimately to death. During their low-grade period, these tumors exhibit systematically a spontaneous and continuous radiological growth, whatever their histological subtypes. The radiological tumor growth is easily quantified by measuring the evolution of the equivalent tumor diameter (calculated from the tumor volume), obtaining the velocity of diametric expansion (VDE). The spontaneous VDE of LGG varies markedly with an average VDE of about 4 mm/year. It depends on intrinsic factors (1p19q codeletion status, P53 overexpression status) and can be modified by extrinsic factors such as pregnancy. VDE has a strong prognostic significance regarding progression-free and overall survivals. Therefore, VDE should be integrated along with the other "static" parameters (multimodal imaging, histological, and molecular analyses) in the initial investigations (Pallud et al. 2012). Assessment of VDE obtained before, during, and after cancer therapy helps in analyzing the effects on an individual basis as a guide to the decision in management.

16.21.1.11 Personalized Therapy of Oligodendroglial Tumors (OTs)

Oligodendroglial tumors (OTs) constitute one-third of gliomas and their distinction from astrocytic gliomas is important for both prognosis and therapy, but is often not adequately accurate. Because response to chemotherapy varies and the adverse effects may outweigh benefits in pathological types of tumors that do not respond to chemotherapy, there is thus an urgent need for refined diagnostic markers to improve glioma classification and predicting their chemosensitivity. LOH markers or in situ

hybridization probes mapping to 1p36 have been used to identify chemosensitive OTs. It has become increasingly clear, however, that not all chemotherapy-sensitive OTs can be identified by this limited set of diagnostic tools and that some OTs, despite their loss of 1p, are chemoresistant. Scientists at the University Medical Center (Nijmegen, the Netherlands) are developing novel predictive diagnostic tools for personalizing the treatment of OTs by aiming to (1) define a molecular profile capable of identifying all procarbazine, lomustine, and vincristine (PCV)-chemosensitive gliomas and (2) identify genes/signaling pathways involved in PCV chemosensitivity.

Anaplastic oligodendroglioma (AO) and anaplastic oligoastrocytoma (AOA) are treated with surgery and radiotherapy (RT) at diagnosis, but they also respond to PCV, raising the possibility that early chemotherapy will improve survival. A randomized clinical trial showed that for patients with AO and AOA, PCV plus RT does not prolong survival. Longer progression-free survival after PCV plus RT is associated with significant toxicity. A significant finding of this trial was that tumors lacking 1p and 19q alleles are less aggressive or more responsive or both (Cairncross et al. 2006). The specific chromosomal change in oligodendroglial brain tumors is thus associated with a very good prognosis and may also identify patients who would benefit from chemotherapy treatment in addition to radiotherapy at diagnosis for long-term tumor control. The findings could change the future of how brain cancers are diagnosed and treatments are personalized based on genetic makeup of the tumor. Testing for chromosomal deletions should be a mandatory part now of the management of patients with these tumors.

Clinical implementation of these results is expected to greatly improve routine glioma diagnostics and will enable a patient-specific therapeutic approach. In order to develop a routine-diagnostic test for chemosensitivity prediction that is widely applicable and cost-effective, an established multiplex ligation-dependent probe amplification (MLPA) assay for OT diagnostics will be revamped by adding novel biomarkers that are identified by a combined array approach. MLPA analysis will be performed on archival, paraffin-embedded tissue of a set from clinically well-documented gliomas, and marker patterns will be identified that correlate with clinical outcome. Protocols will be established that are able to distinguish chemosensitive and chemoresistant tumors, and implementation of these protocols in routine diagnosis will enable tailored chemotherapy for individual glioma patients, thereby avoiding unnecessary harmful side effects and improving their quality of life.

16.21.1.12 Personalized Therapy of Neuroblastomas

Neuroblastoma usually arises in the tissues of the adrenal glands but is also seen in the nerve tissues of the neck, chest, abdomen, and pelvis. It responds to chemotherapy with topotecan, which interacts with a critical enzyme in the body called topoisomerase. This enzyme helps DNA unwind so it can replicate, and topotecan inhibits its function, leading to cell death. However, pinpointing the optimum dosage to treat neuroblastoma can be tricky. Researchers at St. Jude Children's Research Hospital (Memphis, TN) have shown that finding the optimal dosage of the drug

topotecan improves the efficacy of treatment of children with neuroblastoma. From the results of a number of earlier studies, they found that giving a low topotecan dosage on an extended schedule was the best way to destroy tumors. More recently they found that close monitoring and fine-tuning topotecan drug levels for each child by a technique called pharmacokinetic-based (PK-based) dosing improves the response to treatment. PK-based dosing reduces variability in the amount of topotecan in the body, leading to improvements in response and ultimately improving the odds of survival. The aim is to get the right dosage of topotecan for a good antitumor effect and to minimize toxicity. In a prospective phase II trial, topotecan was administered with PK guidance on a protracted schedule to achieve targeted systemic exposure and was found to be active against neuroblastoma (Santana et al. 2005).

The aim of the initial treatment with the drug is to quickly reduce the size of the tumor that must be surgically removed. Reducing tumor size with topotecan and surgery also reduces the risk that the cancer will develop resistance to standard chemotherapy drugs that are administered afterward. The children with PK-guided drug administration did exceedingly well and tolerated the therapy with few ill effects. PK-based topotecan dosing is also being used for the brain tumor medulloblastoma and the eye cancer retinoblastoma. The scientists are now working on a method where they could tell pediatric oncologists that they could adjust the topotecan dosage according to patient characteristics to get a better antitumor effect and not even need to check blood levels. This would be a personalized approach to treatment.

Children with high-risk neuroblastoma have a poor clinical outcome. Vaccination with antigen-loaded dendritic cells (DCs) is being investigated for these children. Loading of DCs with apoptotic neuroblastoma cells or transfection with tumor mRNA represents promising strategies for the development of individualized cancer vaccines/cancer gene therapy in the treatment of neuroblastoma.

16.21.1.13 Personalized Therapy of Medulloblastomas

Medulloblastoma is a malignant tumor of the cerebellum usually diagnosed in children at the median age of 5 years, but it may occur in young adults. Treatment is surgery followed by radiation therapy and chemotherapy, which have serious short-term and long-term adverse effects. Patients with recurrence after primary therapy have a particularly poor prognosis. The hedgehog pathway, an embryonic signaling cascade that regulates stem cell and progenitor cell differentiation, is involved in the pathogenesis as medulloblastoma arises from these cells. PTCH1 is an inhibitory cell surface receptor that constitutively suppresses the activation of the hedgehog pathway by inhibiting smoothened homolog (SMO)—a transmembrane protein that activates the downstream hedgehog signaling pathway. Hedgehog ligands bind to and inactivate PTCH1, derepressing SMO and promoting pathway activation. Activation of hedgehog signaling, usually due to inactivating mutations of PTCH1, has been shown in ~30 % of medulloblastomas in humans.

GDC-0449, an orally bioavailable selective inhibitor of SMO, showed efficacy in phase I trials on patients with advanced basal-cell carcinoma, another type of tumor that is known to harbor PTCH1 mutations. It was used successfully in a patient with

advanced medulloblastoma that had been refractory to multiple prior therapies (Rudin et al. 2009). However, this patient rapidly acquired resistance to GDC-0449. Identifying the mechanisms of acquired resistance to selective hedgehog pathway inhibitors in patients with medulloblastoma will be of particular interest in future studies. The development of a diagnostic biomarker for hedgehog pathway activation has been challenging because alteration of many pathway components may result in an activated phenotype. A gene expression signature that appears to correlate with hedgehog pathway activation in medulloblastoma showed specific pathway activation in this patient's tumor. Testing this and other potential strategies for identifying biomarkers will be important components of future clinical trials of hedgehog pathway inhibitors.

16.21.1.14 Personalized Management of Germ Cell Brain Tumors

A phase II study was carried to determine response to chemotherapy and survival after response-based radiation therapy (RT) in children with CNS germ cell tumors using serum or cerebrospinal fluid (CSF) biomarkers: human chorionic gonadotropin (HCG) and alpha-fetoprotein (AFP) (Kretschmar et al. 2007). Children with germinomas and normal biomarkers received cisplatin + etoposide, alternating with vincristine + CPM, whereas children with nongerminomatous tumors or with abnormal biomarkers received doubled doses of cisplatin and CPM. For germinoma patients in complete response (CR), RT was decreased from but dose was maintained in high-risk patients. Response (germinoma, 91 %; nongerminomatous, 55 %) and survival are encouraging after this regimen plus response-based RT.

16.21.1.15 Personalized Management of Meningiomas

Meningiomas are the most common primary brain tumors and affect ~170,000 patients in the USA. They are usually benign but can turn malignant in about 10 % of cases. Even benign tumors require surgery if they affect the surrounding brain tissue and disrupt neurological functions. Genomic analysis has shown that the entire genetic landscape of meningiomas can be explained by abnormalities in just five genes. Nearly half of atypical meningiomas were neurofibromin 2 (NF2)-mutant, an already known mutation responsible for genomic instability as well as association with malignancy and localizing to the cerebral and cerebellar hemispheres. The other four gene mutations now discovered are (Clark et al. 2013):

1. Mutations in TRAF7, a proapoptotic E3 ubiquitin ligase, were found in nearly one-fourth of all tumors. Meningiomas with these mutations are found in the skull base and are unlikely to become malignant.
2. Recurrent mutation in KLF4, a transcription factor known for its role in inducing pluripotency. It can induce stem cell formation, even in cells that have fully differentiated into a specific tissue type.
3. AKT1(E17K), a mutation known to activate the PI3K pathway and is linked to malignancy.

4. SMO mutations, which activate hedgehog signaling, were identified in ~5 % of non-NF2 mutant meningiomas. These non-NF2 meningiomas were clinically distinctive—nearly always benign, with chromosomal stability, and originating from the skull base. SMO mutations had previously been found in basal-cell carcinoma and are the target of an already approved drug for that form of skin cancer. It is feasible to use targeted chemotherapy on patients with non-NF2 mutations, especially those with recurrent or invasive meningiomas and those who are surgically at high risk. Individualized chemotherapies could also spare patients' irradiation treatment, a risk factor for progression of these generally benign tumors.

Collectively, these findings identify distinct meningioma subtypes, suggesting novel avenues for targeted therapeutics. Tumors mutated with each of these genes tend to be located in different areas of the brain, which can indicate how likely they are to become malignant. Knowledge of the genomic profile of the tumors and their location in the brain make it possible for the first time to develop personalized medical therapies for meningiomas, which currently are managed only surgically.

16.21.2 Personalized Management of Breast Cancer

Personalized management of breast cancer involves improved diagnosis and selection of therapy as well as development of personalized drugs, which are targeted and specific for cancer pathways involved in breast cancer. Ninety percent of patients with early-stage breast cancer can be cured when treated only with radiation and surgery, but another 3 % also require chemotherapy to stop the cancer from spreading elsewhere. The problem is to identify these 3 %. Most patients endure chemotherapy and its devastating side effects, even though for 90 % of them the treatment is unnecessary. Breast cancer was the first cancer where a personalized approach was identified by making a distinction between estrogen receptor-positive and estrogen receptor-negative cancers. Breast cancer can be typed into the following categories with distinct differences in prognosis and response to therapy:

- Estrogen receptor (ER) positive: 65–75 % of breast cancers are ER^+ and are further divided into luminal A and luminal B subtypes.
- HER2 positive constitutes 15–20 % of breast cancer.
- Basaloid type constitutes 15 % of cases and includes those with BRCA1 and P53 mutations.
- Triple negative for ER, progesterone receptors (PRs), and HER2 receptors.

16.21.2.1 Developing Personalized Drugs for Breast Cancer

Developing drugs targeted to pathways involved in breast cancer. Because up to 75 % of breast cancer patients have an abnormality in a specific cell signaling pathway, drugs that target different molecules along that pathway may be especially effective for treating the disease. PI3K pathway is linked to critical growth factor

receptors and is involved in programmed cell death and is aberrant at multiple levels in breast cancer, including mutations in PI3K itself or its many downstream players, such as PTEN or AKT. There is a lot of cross talk between the PI3K pathway and other pathways, a lot of feed-forward and feedback loops. Central nodes between these intersecting circles can be effectively targeted with drugs.

Only one PI3K pathway inhibitor is in use to date, but others are increasingly being developed and tested. At least 20 different companies have recognized the importance of the pathway in breast cancer and are trying to develop drugs that target it.

In the future, breast cancer tissue samples from newly diagnosed patients can be tested for their specific PI3K pathway abnormality in order to find a drug that zeroes in on what may be that particular cancer's vulnerable point. Using those drugs in combination with other treatments such as chemotherapy may significantly advance breast cancer care.

Rational drug design for breast cancer. Capecitabine is an example of a rationally designed cytotoxic treatment. It is designed to generate 5-FU preferentially in tumor cells by exploiting the higher activity of the activating enzyme thymidine phosphorylase in tumors compared with healthy tissues. Tumor-specific activation has the potential to enhance efficacy and minimize toxicity. Proof of this principle is provided by clinical trial results showing that capecitabine is effective and has a favorable safety profile in the treatment of metastatic breast cancer. Breast cancer treatment thus will be determined by tumor biology as well as patient characteristics. Improved molecular characterization and greater understanding of tumorigenesis will enable more individualized treatment.

Developing personalized drugs for triple-negative breast cancer. Triple-negative tumors, i.e., hormone receptor and ERBB2 negative, account for 15 % of all breast cancers and frequently harbor defects in DNA double-strand break repair through homologous recombination, such as BRCA1 dysfunction. Whereas target-specific drugs are available for treating ERBB2-overexpressing and hormone receptor-positive breast cancers, no personalized therapy exists for triple-negative mammary carcinomas. The DNA repair defects characteristic of BRCA1-deficient cells confer sensitivity to poly(ADP-ribose) polymerase 1 (PARP1) inhibition, which could be relevant to the treatment of triple-negative tumors. AZD2281, a PARP inhibitor, was tested in a genetically engineered mouse model (GEMM) for BRCA1-associated breast cancer (Rottenberg et al. 2008). Treatment of tumor-bearing mice with AZD2281 inhibited tumor growth without signs of toxicity, resulting in strongly increased survival. Long-term treatment with AZD2281 in this model resulted in the development of drug resistance, caused by upregulation of Abcb1a/b genes encoding P-glycoprotein efflux pumps, which could be reversed by coadministration of the P-glycoprotein inhibitor tariquidar. Combination of AZD2281 with cisplatin or carboplatin increased the recurrence-free and overall survival, suggesting that AZD2281 potentiates the effect of these DNA-damaging agents. These results demonstrate in vivo efficacy of AZD2281 against BRCA1-deficient breast cancer and illustrate how GEMMs of cancer can be used for preclinical evaluation of novel therapeutics and for testing ways to overcome therapy resistance.

16.21.2.2 Gene Expression plus Conventional Predictors of Breast Cancer

In a retrospective study, researchers combined conventional predictors of breast cancer outcomes—factors such as patient age and tumor size—with information about gene expression profiles in nearly a thousand breast cancer tumor samples (Acharya et al. 2008). Their findings suggest that incorporation of gene expression signatures into clinical risk stratification can refine prognosis and potentially guide treatment of breast cancer. The identification of subgroups may not only refine predictions about patient outcomes but also provide information about the underlying biology and the tumor microenvironment because gene expression patterns reveal different genetic pathways that are activated or silenced in different tumors. Tumors in the high-risk group with the best outcomes tended to have low expression of cancer risk genes, CIN, etc. On the other hand, tumors that have high expression of genes associated with oncogenic pathway activation, wound healing, etc., tend to be associated with poorer outcomes. Genetic signatures within high-, medium-, and low-risk groups were associated with different responses to chemotherapy treatments. Prospective studies are needed to determine the value of this approach for individualizing therapeutic strategies.

Typically, ER-positive tumors, which are more common in older women, can be treated with drugs that inhibit estrogen production. However, not all tumors that start out estrogen receptor positive remain so. Some estrogen receptor-positive tumors respond to antiestrogen therapy at first but eventually become estrogen receptor negative and resistant to these drugs. This transition is associated with patient relapse and poor overall outcomes. During a phase II clinical trial in 2008, a team of researchers at Washington University School of Medicine (St. Louis, MO) was able to classify ER-positive tumors into low-, medium-, and high-risk groups depending on the genetic signature in the tumors a month after patients started treatment. Rather than just looking for the specific gene signature in tumors before treatment, the researchers also tested expression of 50 genes after treatment with letrozole, a drug that blocks estrogen production. The team identified a group of about 10–15 % of estrogen receptor-positive tumors that behave in a completely hormone refractory way. This approach can predict which seemingly low-risk tumors are destined to become high risk and help guide treatment accordingly. This new knowledge may eventually change the way that physicians design estrogen receptor-positive breast cancer therapies. For example, it may be possible to target aggressive post-surgery chemotherapy to those with higher-risk tumors.

Earlier studies at NCI using mouse models and human breast cancer populations have shown that metastasis susceptibility is an inherited trait. This same combined approach facilitated the identification of a number of candidate genes that, when dysregulated, have the potential to induce prognostic gene expression profiles in human datasets. A further series of expression profiling experiments in a mouse model of metastatic breast cancer have shown that both the tumor epithelium and invading stromal tissues contribute to the development of prognostic gene signatures (Lukes et al. 2009). Furthermore, analysis of normal tissues and tumor transplants suggests that prognostic signatures result from both somatic and inherited components, with the inherited components being more consistently predictive.

EGFR and the ER are the most dangerous combination of molecules overproduced in breast cancer. When both are overfunctioning, patients are resistant to therapy and die quickly of disease progression. A study has shown how the master gene called SRC-3 (steroid receptor coactivator 3) not only enhances estrogen-dependent growth of cancer cells by activating and encouraging the transcription of a genetic message into a protein but also sends a signal to the activating enzyme called FAK (focal adhesion kinase) found on the cell's membrane to promote cell motility or movement, which is a key element of cancer spread or metastasis (Long et al. 2010). Overexpression of SRC-3 is found in two-thirds of breast cancers. This study shows that SRC-3 can produce an alternative form of its coactivator protein—a shorter form that is missing the part of the protein (exon) that keeps it in the nucleus. With that portion gone, it leaves the nucleus and goes into the cytoplasm and travels to the membrane, where the enzyme PAK1 (p21-activated kinase 1) phosphorylates SRC-3, enabling it to function at the membrane.

16.21.2.3 Her2 Testing in Breast Cancer as a Guide to Treatment

The information provided by a personal genetic test might be of real value in identifying the woman whose risk for breast cancer or other cancers is likely to be amplified by oral contraceptives. Depending on the mutation, oral contraceptives can increase the risk of breast cancer and may also fail to protect against ovarian cancer. Thus, a positive test for certain genetic mutations means that the strategy of using oral contraceptives to reduce the risk of ovarian cancer should be abandoned. In contrast, a woman worried about ovarian cancer who does not have one of these hereditary contraindications could then take oral contraceptives without danger of precipitating a known hereditary breast cancer.

Women with a family history of breast cancer also have the option for prophylactic breast removal, which reduces the breast cancer risk by 90 %. Chemoprevention with tamoxifen or other agents is another option. The goal is to make chemoprevention as effective as prophylactic mastectomy.

There is evidence that some of the gene mutations in breast cancer are relevant to treatment. The human epidermal growth factor receptor-2 (HER2) gene also known in avian species as c-erbB-2 (avian *er*ythro*b*lastic leukemia viral oncogene homolog 2) or in the rat as neu (*neu*roblastoma oncogene) is amplified in 20–30 % of breast cancers. HER2 gene amplification and HER2 overexpression occur early in the development of breast cancers and are found in a high proportion of ductal carcinomas in situ (DCIS), noninvasive cancers that generally do not give rise to metastases. In DCIS, HER2 overexpression is found specifically in poorly histologically differentiated disease and not in well-differentiated cancers. HER2 expression is associated with response to TCH (Herceptin) and its lack with resistance to therapy. Benefit of anti-HER2 therapies demonstrated in clinical trials indicates that HER2 is, to date, one of the most promising molecules for targeted therapy. Nevertheless, since tumor cells utilizing alternative growth signaling pathways through transmembrane receptors as well as intracellular signaling transduction molecules can bypass HER2 blockade, a future ambitious aim is the successful combination of

anti-HER2 strategies with drugs directed to molecules that contribute to anti-HER2 resistance (Tagliabue et al. 2010).

Various methods that have been used to analyze the HER2 status of a tumor include the following:

- IHC: protein expression levels
- ELISA: shedding of HER2 receptor
- FISH: HER2 gene amplification
- Quantitative PCR: HER2 gene amplification
- Quantitative RT-PCR: mRNA expression level

In practice, IHC is the most frequently used method. However, it is recommended that all specimens with weakly positive IHC (+2 HercepTest result) be evaluated by FISH for HER2/neu gene amplification. The results of both assays should be considered before making a decision to recommend anti-HER2 therapy. The LightCycler™ PCR assay (Roche) has now been developed specifically to assess HER2 gene amplification. The advantages are:

- It is accurate for determining HER2 gene amplification and correlates well with FISH: 85 % sensitivity and 95 % specificity.
- It is a rapid screening method with up to 30 samples per run.
- The kit uses a reference sequence on chromosome 17 so that a correct data interpretation should be possible in polysomic cases.

One limitation of LightCycler PCR is that it does not give histopathological assignment. Microdissection may be required in critical cases. The combined use of LCM, DNA microarray, and real-time quantitative PCR technologies now provides a unique opportunity to elucidate the in vivo genetic events that underlie the initiation and progression of human breast cancer. The clinical utility of the serum test as a prognostic indicator has not yet been fully established but is under investigation.

Current methods for checking HER2 are problematic because of issues with intra- and interlaboratory reproducibility and preanalytic variables, such as fixation time. In addition, the commonly used HER2/chromosome 17 ratio presumes that chromosome 17 polysomy is present when the centromere is amplified, even though analysis of the rest of the chromosome is not included in the assay. In one study, 97 frozen samples of invasive lobular and invasive ductal carcinoma, with known ICH and FISH results for HER2, were analyzed by aCGH to a commercially available bacterial artificial chromosome whole-genome array containing 99 probes targeted to chromosome 17 and the HER2/TOP2 amplicon (Yeh et al. 2009). Results were 97 % concordant for HER2 status, meeting the College of American Pathologists/American Society of Clinical Oncology's validation requirements for HER2 testing. No case of complete polysomy 17 was detected even though multiple breast cancer cases showed polysomies of other chromosomes. Therefore, aCGH is an accurate and objective DNA-based alternative for clinical evaluation of HER2 gene copy number, and that polysomy 17 is a rare event in breast cancer. It is commercially available as HerScan™ (CombiMatrix Molecular Diagnostics).

16.21.2.4 HER2/neu-Derived Peptide Vaccine for Breast Cancer

HER2-positive breast cancers, which contain more receptors than is typical, are found in 20–30 % of all breast cancer patients and are treated by TCH and lapatinib that latch onto these receptors and destroy them. However, some of these patients develop resistance to these therapies or develop cancer metastasis, which are hard to treat. Vaccination is an attractive alternative approach to provide HER-2/neu-specific antibodies and may in addition concomitantly stimulate HER2-reactive T cells. A pilot clinical trial has shown that HER2-pDNA vaccination in conjunction with GM-CSF and interleukin-2 administration is safe, well tolerated and can induce long-lasting cellular and humoral immune responses against HER2 in patients with advanced breast cancer (Norell et al. 2010).

HER2/neu, a source of immunogenic peptides, is expressed in >75 % of breast cancer patients. Clinical trials have been conducted with the HER2/neu E75 peptide vaccine in breast cancer patients. Results show that most patients with various levels of HER2/neu expression respond immunologically and seem to benefit from vaccination (Benavides et al. 2009). E75 is predicted to bind to HLA-A3, and preclinical data support this. Another trial has demonstrated that HLA-A3 patients respond similarly to E75 vaccination as HLA-A2 patients, suggesting the potential use of the E75 vaccine in up to 76 % of the population (Patil et al. 2010). Antigen Express is developing a peptide immunotherapeutic for patients with HER-2/neu-positive breast cancer, which is currently in phase II clinical trials.

16.21.2.5 Molecular Diagnostics in Breast Cancer

Methods of molecular diagnosis of breast cancer were described in Chap. 6. A few points that are important for applications in personalized medicine are described here.

Early detection of metastases. Detection of CTCs before initiation of first-line therapy in patients with metastatic breast cancer is highly predictive of overall and progression-free survival. This can aid appropriate patient stratification and design of tailored treatments.

16.21.2.6 Pharmacogenetics of Breast Cancer

Polymorphisms in tamoxifen-metabolizing genes affect the plasma concentration of tamoxifen metabolites. In a study, CYP450 2D6 and CYP3A5 genotype were determined from paraffin-embedded tumor samples and buccal cells (living patients) in tamoxifen-treated women enrolled onto a North Central Cancer Treatment Group adjuvant breast cancer trial (Goetz et al. 2005). In tamoxifen-treated patients, women with the CYP2D6 *4/*4 genotype tend to have a higher risk of disease relapse and a lower incidence of hot flashes.

16.21.2.7 Proteomic-Based Personalized Management of Breast Cancer

Despite recent advances in breast cancer therapy, women with similar types of breast cancers may respond very differently to standard treatments. The emerging field of clinical proteomics has the potential to revolutionize breast cancer therapy. The ultimate goal of clinical proteomics is to characterize information flow through protein cascades for individual patients. After the protein networks have been elucidated, drug therapies may be specially designed for each patient. Proteomic technologies of LCM and reverse-phase protein arrays (RPPAs) enable scientists to analyze relative abundances of key cellular signaling proteins from pure cell populations. Cell survival and apoptotic protein pathways are currently being monitored with LCM and RPPAs at the National Institutes of Health (NIH) in phase II clinical trials of metastatic breast and ovarian cancers. Ultimately, proteomics will become an integral component of tracking and managing personalized breast cancer therapy.

Examination by 2D GE and MS of nipple aspirate protein samples taken from a group of patients who had been diagnosed with unilateral primary invasive ductal breast carcinoma and also had an apparently normal contralateral breast has revealed differential expression patterns of ductal fluid proteins, some evidence of known and possibly new biomarkers and drug targets for breast cancer. The patient-to-patient variability of these differences may reflect variables in the disease structure and may prove to be of clinical diagnostic and therapeutic significance to individual patients. For example, the presence or absence of known biomarkers detected in the differences in the fluids can be used to determine the aggressiveness of the cancer (e.g., the presence or level of cyclin E) or signal the appearance of a cancer-related genetic instability or hereditary component (e.g., the absence or level of BRCA1). However, this approach requires clinical trials for comparison with the gold standards such as mammograms, ultrasound, biopsy, nipple lavage and aspirate cytology, and serum markers. The presence of known drug targets detected in the differences in the fluids may also be used in the future to indicate what drugs to use.

16.21.2.8 Predicting Response to Chemotherapy in Breast Cancer

Breast cancer patients have benefited from the use of targeted therapies directed at specific molecular alterations. Some of the methods used to identify various pathways or overexpression of some genes for predicting response to therapy are:

Predicting response to trastuzumab treatment. TCH is active against the overexpressed HER2 oncogene in breast cancer, and several prospective, randomized trials have shown that adjuvant TCH substantially reduces rates of recurrence and death in patients with early-stage disease. Combined therapy of TCH with anthracycline (AC-T)-based regimens has been associated with cardiac toxicity. A randomized trial showed that the addition of 1 year of adjuvant TCH significantly improved disease-free and overall survival among women with HER2-positive breast cancer (Slamon et al. 2011). The risk–benefit ratio favored the nonanthracycline TCH

regimen over AC-T plus TCG, given its similar efficacy, fewer acute toxic effects, and lower risks of cardiotoxicity and leukemia.

A DNA probe for the HER2 gene is used to predict whether a breast cancer patient is a candidate for TCH treatment. Current medical practice requires that all patients who are considered for TCH treatment be tested for HER2 amplification or overexpression. SPOT-Light® HER2 CISH Kit (Life Technologies), which is approved by the FDA, is based on chromogenic in situ hybridization (CISH), where results are visualized under a standard bright-field microscope, as opposed to FISH tests, in which the results must be visualized using a fluorescent microscope. This specialized microscope frequently requires that the analysis is done at a reference lab. In addition, HER2 CISH test results are quantifiable, removing the subjectivity inherent in tests based on IHC.

Genomic predictor of response to taxane–anthracycline chemotherapy. A prospective multicenter study was conducted to test genomic predictors for chemotherapy containing sequential taxane- and anthracycline-based regimens in patients with newly diagnosed ERBB2 (HER2 or HER2/neu)-negative breast cancer (Hatzis et al. 2011). Breast cancer treatment sensitivity was predicted using combination of signatures for (1) sensitivity to endocrine therapy, (2) chemoresistance, and (3) chemosensitivity, with independent validation and comparison with other reported genomic predictors of chemotherapy response. A genomic predictor combining ER status, predicted chemoresistance, chemosensitivity, and endocrine sensitivity and identified patients with high probability of survival following taxane and anthracycline chemotherapy.

Use of PET to determine response to chemotherapy. In patients with metastatic breast cancer, sequential 18F-FDG PET enables prediction of response to treatment after the first cycle of chemotherapy. The use of 18F-FDG PET as a surrogate endpoint for monitoring therapy response offers improved patient care by individualizing treatment and avoiding ineffective chemotherapy.

Prediction of response to paclitaxel. Breast cancers show variable sensitivity to paclitaxel. Tubulin polymerization assay has been used to show that low tau expression renders microtubules more vulnerable to paclitaxel and makes breast cancer cells hypersensitive to this drug. Low tau expression, therefore, may be used as a biomarker to select patients for paclitaxel therapy. Inhibition of tau function by RNAi might be exploited as a therapeutic strategy to increase sensitivity to paclitaxel.

Predicting the response to antiestrogen drugs. According to the NCI, about two-thirds of women with breast cancer have estrogen receptor (ER)-positive breast cancer, in which tumor growth is regulated by the natural female hormone estrogen. Estrogen is known to promote the growth of most types of breast cancer. However, another gene, the retinoblastoma tumor suppressor (RB) gene, is functionally inactivated in the majority of human cancers and is aberrant in one-third of all breast cancers. RB regulates G1-/S-phase cell cycle progression and is a critical mediator of antiproliferative signaling. RB deficiency compromises the short-term cell cycle inhibition following cisplatin, ionizing radiation, and antiestrogen therapy of breast cancer with drugs such as tamoxifen (Bosco et al. 2007). Specific analyses of an RB

gene expression signature in human patients indicate that deregulation of this pathway is associated with early recurrence following tamoxifen monotherapy. Thus, because the RB pathway is a critical determinant of tumorigenic proliferation and differential therapeutic response, it may represent a critical basis for directing therapy in the treatment of breast cancer. The RB tumor suppressor can be used as a biomarker for how tumors will respond to antiestrogen therapy and could become the basis for deciding how patients with ER-positive breast cancer are treated clinically. This is a way to predict when antiestrogen drug therapies are inappropriate for patients with hormone-dependent breast cancer so that physicians can immediately begin treating the patient with alternative drugs that are more likely to succeed. However, comprehensive clinical research is needed before this new method for predicting the success of antiestrogen drugs is applied in daily patient care.

Role of p63/p73 pathway in chemosensitivity to cisplatin. Breast cancers lacking estrogen and progesterone receptor expression and Her2 amplification exhibit distinct gene expression profiles and clinical features, and they comprise the majority of BRCA1-associated tumors. Global GEP has uncovered previously unrecognized subsets of human breast cancer, including the “triple-negative” or “basal-like” subset characterized by a lack of ER and progesterone receptor (PR) expression, the absence of HER2 amplification, and the expression of basal epithelial markers. Triple-negative breast cancers (TNBCs) are the most common subtype arising in patients harboring germ line mutations in the breast cancer predisposition gene breast cancer 1, early onset (BRCA1). Both BRCA1-associated and the more common sporadic triple-negative tumors share similar gene expression profiles and both are refractory to commonly used chemotherapeutic agents and as a result are associated with a relatively poor prognosis. The p53 family member p63 controls a pathway for p73-dependent cisplatin sensitivity specific to these “triple-negative” tumors. A study shows that p63 is a survival factor in a subset of breast cancers and provides a novel mechanism for cisplatin sensitivity in these triple-negative cancers and suggests that such cancers may share the cisplatin sensitivity of BRCA1-associated tumors (Leong et al. 2007).

Targeted therapy of breast cancer with AGTR1 antagonists. To identify additional opportunities for targeted therapy, a study searched for genes with marked overexpression in subsets of tumors across a panel of breast cancer profiling studies comprising 3,200 microarray experiments (Rhodes et al. 2009). In addition to prioritizing ERBB2, the researchers found AGTR1, the angiotensin II receptor type I, to be markedly overexpressed in 10–20 % of breast cancer cases across multiple independent patient cohorts. Validation experiments confirmed that AGTR1 is highly overexpressed, in several cases more than 100-fold. AGTR1 overexpression was restricted to estrogen receptor-positive tumors and was mutually exclusive with ERBB2 overexpression across all samples. Ectopic overexpression of AGTR1 in primary mammary epithelial cells, combined with angiotensin II stimulation, led to a highly invasive phenotype that was attenuated by the AGTR1 antagonist losartan. Similarly, losartan reduced tumor growth by 30 % in AGTR1-positive breast cancer xenografts. Taken together, these observations indicate that marked AGTR1 overexpression

defines a subpopulation of ER-positive, ERBB2-negative breast cancer that may benefit from targeted therapy with AGTR1 antagonists, such as losartan.

NQO1 enzyme-based test for response to anthracycline chemotherapy. NQO1 enzyme was shown in a Helsinki University study to protect cells against oxidative stress, and patients having one variant of the protein, NQO1*2, had worse survival chances when they were treated with an anthracycline-based chemotherapy compared with an alternative therapy. Women in the study who possessed a double copy of the NQO1*2 variant in their genome had only a 17 % survival rate while those with only a single copy or without the variant had a survival rate of 75 %. DNA Repair Company has licensed the exclusive North American rights to a test from Helsinki University and plans to use a variant of the NQO1 enzyme to create personalized medicine tests.

Preoperative Endocrine Prognostic Index (*PEPI Score*). The PEPI score is a new predictive measurement that could help many women diagnosed with early-stage breast cancer avoid chemotherapy after surgery by identifying them as having little risk of a relapse (Ellis et al. 2008). About 83 % of patients are cured of breast cancer, but 17 % are resistant to current treatments. The PEPI score was derived from tumor characteristics present after women with stage 2 and 3 breast cancer underwent 4 months of antiestrogen therapy before having breast surgery. The PEPI score considers the size of the breast tumor, whether cancer is present in nearby lymph nodes, how fast tumor cells are multiplying, and whether tumors lose their estrogen receptors. Women with a PEPI score of 0 had almost no risk of cancer recurrence during the 5-year follow-up. They could safely avoid taking chemotherapeutic agents after surgery. Women with PEPI scores of 4 or above are at very high risk of having their cancer return and should be given all appropriate postsurgical treatments.

Decreased breast density as a biomarker of response to tamoxifen. Increased breast density on mammography is the leading risk factor for breast cancer, apart from age. The International Breast Intervention Study I (IBIS-I), a trial of tamoxifen for ER-positive breast cancer prevention conducted at the Cancer Research UK Centre for Epidemiology, Mathematics and Statistics in London, has shown that a reduction in breast density of at least 10 % may predict who benefits from the breast cancer preventive effects of tamoxifen. Those with reduced breast density after 12–18 months of treatment had a 52 % reduced risk of breast cancer. By contrast, those women who did not have a decrease in breast density had only an 8 % risk reduction.

Measurement of estrogen receptor mRNA to predict response to tamoxifen. Quantification of mRNA has historically been done by RT-PCR. A robust method of detection of mRNA utilizing ISH has been described that is linear and shows high specificity with low background. AQUA method of quantitative immunofluorescence (QIF) has been tested for measuring mRNA in situ using ESR1 alpha gene in breast cancer to determine its predictive value compared to ER protein (Bordeaux et al. 2012). mRNA for ER (ESR1) and ubiquitin C (UbC) were visualized using RNAscope probes and levels were quantified by quantitative ISH (qISH) on two

Yale breast cancer cohorts on tissue microarrays. ESR1 levels were compared to ER protein levels measured by QIF using the SP1 antibody. Results showed that ESR1 mRNA is reproducibly and specifically measurable by qISH on tissue collected from 1993 or later. ESR1 levels were correlated to ER protein levels in a nonlinear manner on two Yale cohorts. High levels of ESR1 were found to be predictive of response to tamoxifen in a manner different from value of ER.

Prediction of response to chemotherapy by intrinsic subtypes. A 50-gene subtype predictor was developed using microarray and quantitative RT-PCR to improve on current standards for breast cancer prognosis and prediction of chemotherapy (Parker et al. 2009). It incorporates the gene expression-based intrinsic subtypes, luminal A, luminal B, and HER2-enriched, which are generally considered types with a poor prognosis. Breast cancer experts also typically identify a fifth breast cancer type known as normal-like. The 50-gene set also recognizes the normal-like type, but instead of being a fifth type of breast cancer, the normal-like classification is an indicator that a sample contains insufficient tumor cells to make a molecular diagnosis and that a new sample needs to be taken.

The genetic test was highly sensitive and very predictive for chemotherapy response. The test was more predictive than typically used clinical molecular markers such as estrogen receptor status, progesterone receptor status, or HER2 gene expression status. Luminal A was found to be not sensitive to the chemotherapy, suggesting that patients with this good-prognosis type can forgo chemotherapy in favor of hormone-based therapy. Among the poor-prognosis tumor types, basal-like breast cancer was the most sensitive to the chemotherapy and luminal B the least.

Diagnosis by intrinsic subtype adds significant prognostic and predictive information to standard parameters for patients with breast cancer. The prognostic properties of the continuous risk score will be of value for the personalized management of node-negative breast cancers. The subtypes and risk score can also be used to assess the likelihood of efficacy from neoadjuvant chemotherapy. This new genomic test is broadly applicable for all women diagnosed with breast cancer. Their 50-gene set can be assayed in preserved tumor samples left over from standard diagnostic procedures, so that tumor samples from breast cancer cases going back a decade or more can be studied. Since the patients in these cases have already been treated, the researchers can quickly discover how well various therapies worked for each breast cancer type. The genomic test technology will be distributed through University Genomics, a company co-owned by the Washington University, the University of Utah, and the University of North Carolina.

Subtyping breast cancer to predict response to chemotherapy. Breast cancers are comprised of molecularly distinct subtypes that may respond differently to pathway-targeted therapies now under development. Collections of breast cancer cell lines mirror many of the molecular subtypes and pathways found in tumors, suggesting that treatment of cell lines with candidate therapeutic compounds can guide the identification of associations between molecular subtypes, pathways, and drug response (Heiser et al. 2012). In a test of 77 therapeutic compounds, the authors found that nearly all drugs showed differential responses across these cell lines, and approximately

one-third showed subtype-, pathway-, and/or genomic aberration-specific responses. These observations suggest mechanisms of response and resistance and may facilitate efforts to develop molecular assays that predict clinical response.

16.21.2.9 Prediction of Resistance to Chemotherapy in Breast Cancer

It is well known that some breast tumors acquire altered genes or chromosomes during the course of treatment that make them resistant to many cancer drugs. With a few exceptions, e.g., ER-sensitive HER2-positive cancer, no tests are done before treatment begins to predict who is going to be resistant or sensitive to chemotherapy. Most breast cancer patients are initially given the same drugs. In search of genetic alterations that might explain disease recurrence despite treatment with adjuvant chemotherapy in some breast cancer patients, scientists at Dana-Farber Cancer Institute (Boston, MA) scanned the genome of stored breast cancer samples from patients who had been treated according to modern guidelines, including the use of anthracyclines. The use of integrated genomics enabled the identification of a small number of overexpressed and amplified genes from chromosome 8q22 that are associated with early disease recurrence despite anthracycline-based adjuvant chemotherapy (Li et al. 2010). The association was confirmed in an analysis of multiple independent cohorts. siRNA-mediated knockdown of either of the two of these genes, the antiapoptotic gene YWHAZ and a lysosomal gene LAPTM4B, sensitized tumor cells to anthracyclines, and overexpression of either of the genes induced anthracycline resistance. Overexpression of LAPTM4B resulted in sequestration of the anthracycline doxorubicin, delaying its appearance in the nucleus. Overexpression of these two genes was associated with poor tumor response to anthracycline treatment in a neoadjuvant chemotherapy trial in women with primary breast cancer. These results suggest that 8q22 amplification and overexpression of LAPTM4B and YWHAZ contribute to de novo chemoresistance to anthracyclines and allow metastatic recurrence. Overexpression of these two genes may predict anthracycline resistance and influence selection of chemotherapy. These findings could lead to a genetic test of breast cancers to help physicians choose the best initial treatment for an individual patient that is less likely to lead to development of resistance. Such a test should not be difficult to develop and could be available for clinical testing in the near future. Testing prior to start of chemotherapy would help to personalize the treatment and reduce the possibility of development of resistance.

The 78-kDa glucose-regulated protein (GRP78), widely used as an indicator of the unfolded protein response (UPR), is induced in the tumor microenvironment. In vitro studies suggest that GRP78 confers chemoresistance to topoisomerase inhibitors, such as Adriamycin (doxorubicin), used for the treatment of breast cancer. In a retrospective study of breast cancer patients who were treated with Adriamycin, archival tumor specimens were analyzed, and the relationship of GRP78 expression level to “time to recurrence” (TTR), used as a surrogate biomarker for drug resistance, was examined (Lee et al. 2006). The data show that 67 % of the study subjects expressed high level of GRP78 in their tumors before the initiation of chemotherapy

and suggest an association between GRP78 positivity and shorter TTR. The use of GRP78 as a predictor for chemoresponsiveness and the potential interaction of GRP78 and/or the UPR pathways with taxanes warrant larger studies.

An experimentally derived IFN-related DNA damage resistance signature (IRDS) is associated with resistance to chemotherapy and/or radiation across different cancer cell lines (Weichselbaum et al. 2008). The IRDS genes STAT1, ISG15, and IFIT1 all mediate experimental resistance. Clinical analyses reveal that $IRDS^+$ and $IRDS^-$ states exist among common human cancers. In breast cancer, a seven-gene-pair classifier predicts for efficacy of adjuvant chemotherapy and for local–regional control after radiation. By providing information on treatment sensitivity or resistance, the IRDS improves outcome prediction when combined with standard markers, risk groups, or other genomic classifiers.

16.21.2.10 Prediction of Adverse Reaction to Radiotherapy in Breast Cancer

Radiotherapy is a very important treatment for breast cancer, but a small number of patients can develop severe side effects. Although fibrosis, telangiectasia, and atrophy all contribute to late radiation injury, they have distinct underlying genetic and radiobiological causes. Fibrosis risk is associated with an inflammatory response, whereas telangiectasia is associated with vascular endothelial cell damage. There is no test at present for an abnormal reaction to radiotherapy. A combined analysis of two UK breast cancer patient studies shows that 8 % of patients are homozygous for the TGF-β1 (C-509T) variant allele and have a 15-fold increased risk of fibrosis following radiotherapy (Giotopoulos et al. 2007). Atrophy is associated with an acute response, but the genetic predisposing factors that determine the risk of an acute response or atrophy have yet to be identified. The identification of the two genes associated with adverse reaction to cancer treatment means that patients who might react badly to radiotherapy could be warned in advance or alternative treatments can be sought. Further work needs to be done as the genes responsible for redness and peeling of the skin during treatment have not been found.

16.21.2.11 Prediction of Recurrence in Breast Cancer for Personalizing Therapy

To tailor local treatment in breast cancer patients, there is a need for predicting ipsilateral recurrences after breast-conserving therapy. After adequate treatment (excision with free margins and radiotherapy), young age and incompletely excised extensive intraductal component are predictors for local recurrence. GEP (wound-response signature, 70-gene prognosis profile (Agendia's MammaPrint test), and hypoxia-induced profile) can identify subgroups of patients at increased risk of developing a local recurrence after breast-conserving therapy.

Lymph node status at the time of diagnosis of breast cancer is considered to be the most important measure for future recurrence and overall survival. It is an imperfect method because a third of patients with no detectable lymph node involvement will develop recurrent disease within 10 years. DNA microarray analysis of primary breast tumors and classification to identify a gene expression signature is strongly predictive of a short interval to distant metastases in patients without tumor cells in local lymph nodes at the time of diagnosis. The poor-prognosis signature consists of genes regulating cell cycle, invasion, metastasis, and angiogenesis. This gene expression profile will be superior to the currently used clinical parameters in predicting disease outcome and selection of patients who would benefit from adjuvant therapy. The ability to accurately predict long-term recurrence with microarrays, however, might prove very important if subsets of patients who will not relapse can be spared the toxicity of adjuvant chemotherapy.

TargetPrint® (Agendia). This FDA-approved test enables quantitative determination of gene expression levels of the estrogen receptor, progesterone receptor, and HER2 in breast cancer biopsies. This is of paramount importance in planning treatment of breast cancer patients after surgery and assists physicians and patients in making informed treatment decisions. TargetPrint runs on Agendia's high-density chip.

Oncotype DX™ Breast Cancer Assay (Genomic Health Inc.). This, a clinically validated multigene RT-PCR test, is available for use in clinical practice to quantify the likelihood of breast cancer recurrence for an individual patient. The assay, performed using FFPE tissue, analyzes the expression of a panel of 21 genes using RT-PCR. The likelihood of distant recurrence in patients with estrogen receptor-positive breast cancer without the involvement of lymph nodes is poorly defined by clinical and histopathological measures. Analysis of RT-PCR profiles obtained from tumor blocks shows that recurrence score is predictive of overall survival in individual tamoxifen-treated patients with node-negative, estrogen receptor-positive breast cancer. In 2007, the American Society of Clinical Oncology recommended using Oncotype DX in its updated guidelines for using breast cancer tumor markers, and it is included in the National Comprehensive Cancer Network's 2008 Breast Cancer Treatment Guidelines.

The MammaPrint test (Agendia). This FDA-approved 70-gene microarray assay is used to provide important prognostic information for individuals with primary invasive breast cancer with lymph node-negative disease of either positive or negative estrogen receptor status. The microarray assay looks at what specific genes are expressed in a patient's tumor. When compared to clinical factors currently used by physicians in the prognosis of breast cancer such as age, tumor size, lymph node status, tumor grade, and estrogen receptor status, the MammaPrint test has shown to provide the best single prognostic information concerning the development of distant metastases. Large-scale prospective clinical trials of the breast cancer prognosis test have been carried out. MammaPrint test outperformed the clinicopathologic risk assessment in predicting all endpoints and adds independent prognostic

information to clinicopathologic risk assessment for patients with early breast cancer as well (Buyse et al. 2006). To facilitate its use in a diagnostic setting, the 70-gene prognosis profile was translated into a customized MammaPrint containing a reduced set of 1,900 probes suitable for high-throughput processing. RNA of 162 patient samples from two previous studies was subjected to hybridization to this custom array to validate the prognostic value. Classification results obtained from the original analysis were then compared to those generated using the algorithms based on the custom microarray and showed an extremely high correlation of prognosis prediction between the original data and those generated using the custom mini-array. Therefore, the array is an excellent tool to predict the outcome of disease in breast cancer patients.

TOP2A FISH pharmDx test (Dako). This uses FISH to detect or to confirm abnormalities in the topoisomerase 2 alpha gene, which is involved in DNA replication. Changes in this gene in breast cancer cells can be used to predict the likelihood of tumor recurrence or long-term survival of a patient. The FDA has approved this test with the remark that this is the first test to be approved that targets the TOP2A gene in cancer patients. The FDA has deemed the test suitable for premenopausal patients or those who have other indicators of higher chances of tumor recurrence, such as tumor size or lymph node involvement, or decreased survival. The test was studied in Danish patients who were treated with chemotherapy after removal of breast cancer tumors. That study used data from tumor samples and clinical data from 767 patients with high-risk tumors, and it confirmed that the test was useful in estimating recurrence and survival in women who had received chemotherapy. Dako received the CE mark for the test in 2007 and has since launched the assay in Europe and the USA.

16.21.2.12 Prognostic Tests for Breast Cancer

A study has demonstrated that a history of hypertension, ER/PR status, HER2 status, metastasis-free interval, metastatic location (including brain, bone, and liver), and BMI at diagnosis with metastatic breast cancer were the most relevant prognostic factors for survival after metastatic disease diagnosis (Jung et al. 2012). Findings of this study may form a foundation for the growing body of knowledge explaining the outcome differences in the treatment of patients with metastatic breast cancer, potentially helping to create tailored counseling and personalized treatment approaches for this vulnerable group.

Prognostic testing of all patients prior to treatment aligns with standard medical practice to distinguish patients by hormone status This information can also enable pharmaceutical companies to clearly define patient stratification that improves clinical trial timelines and outcomes.

Exagen's breast cancer prognostic marker assays. These are the first and only tests to enable specific testing for hormone receptor (including estrogen receptor and progesterone receptor)-positive and for hormone receptor-negative patients using an

improved FISH assay. These prognostic tests separate patients with good prognosis from those with poor prognosis by testing each patient's tumor tissue to detect changes in DNA (e.g., gene copy number) in order to directly reflect changes in the tumor. Exagen's prognostic tests are uniquely developed as separate sets of DNA markers to identify prognosis in hormone-positive and hormone-negative patients, respectively. Both marker sets represent the first prognostic tests that can be used by any FISH-testing laboratory, enabling fit of this testing approach with standard hormone testing prior to treatment. Exagen's small, prognostic marker sets combine to form a testing panel that differs from other existing sets of 20- to 70-gene markers by enabling:

- Use of improved FISH technology with a small (3–5) number of probes to fit with current laboratory testing practices and equipment
- Testing of all breast cancer patients to provide additional prognostic information based on hormone receptor status (including estrogen receptor and progesterone receptor) prior to treatment
- Detection and visualization of tumor-based cellular changes to define only those DNA changes that are specific to tumor tissue

Prognostic gene biomarkers of breast cancer. Three genes, homeobox 13 (HOXB13), interleukin-17B receptor (IL17BR), and CHDH, and the HOXB13:IL17BR ratio index in particular, strongly predict clinical outcome in breast cancer patients receiving tamoxifen monotherapy. A tumor bank study demonstrated that HOXB13:IL17BR index is a strong independent prognostic factor for ER^+ node-negative patients irrespective of tamoxifen therapy (Ma et al. 2006). As a result of this study, these two biomarkers serve as the foundation of the AviaraDx Breast Cancer Profiling Technology.

Activity of a gene, Dachshund (DACH1), which normally regulates eye development and development of other tissues, commandeers cancer-causing genes and returns them to normal. DACH1 inhibits the expression of the cyclin D1 gene, an oncogene that is overexpressed in about half of all breast cancers. Analysis of over 2,000 breast cancer patients has demonstrated that DACH1 correlates with tumor size, stage, and metastasis, with its expression greatly reduced in metastatic breast cancer cells, but increased nuclear DACH1 expression predicts improved patient survival (Wu et al. 2006). The average survival was almost 40 months longer in women in whom their breast cancer continued to express DACH1. DACH1 gene reverts the cancerous phenotype, thus turning the cell back to a premalignant state, and it could be used as a prognostic marker for breast cancer. Other cell fate-determining genes are being examined in an attempt to identify new therapeutics for breast cancer and metastasis.

Researchers at Fox Chase Cancer Center (Philadelphia, PA) have identified an important gene, CEACAM6 (carcinoembryonic antigen-related cell adhesion molecule 6), which is involved in the spread of breast cancer that has developed resistance to long-term estrogen deprivation. The gene may prove to be a useful marker for predicting which patients have the greatest risk of breast cancer recurrence so their physicians can offer the most appropriate treatment plan. The research focused

on breast cancer cells that had grown resistant to aromatase inhibitors (AIs), antihormone drugs to shut down the enzyme aromatase, which lets the body produce estrogen outside the ovaries. These drugs represent one of the most effective forms of hormone therapy for postmenopausal women whose breast cancer tests positive for estrogen receptors, which means that estrogen in the body fuels the growth of cancer cells. Unfortunately, one of the drawbacks to extended use of an AI may be that some of the cancer cells develop resistance to the drug and are able to grow and spread independent of estrogen. Several AI-resistant breast cancer cell lines were developed in the laboratory and found to be very invasive compared to AI-sensitive breast cancer cells. Analyses of gene activity in these AI-resistant cells showed that they express high levels of genes associated with invasiveness and metastasis. However, this aggressive behavior could be reversed by using siRNAs to knock out the CEACAM6 gene. This gene might be an important biomarker for metastasis and a possible target for novel treatments for patients with metastatic breast cancer.

ER-negative basal breast cancer is a heterogeneous disease with at least four main subtypes. It has been shown that the heterogeneity in the clinical outcome of ER^- breast cancer is related to the variability in expression levels of complement and immune response pathway genes independently of lymphocytic infiltration (Teschendorff et al. 2007).

Multigene expression prognostic constellation (*Celera*). The prognostic constellation provides information that is distinct from that predicted by routine clinical assessment tools, such as tumor grade, and can quantify risk for metastasis for variable time periods rather than only categorically for 5 or 10 years. A previously developed 14-gene metastasis score that predicts distant metastasis in breast cancer research subjects without systemic treatment has now been applied to tamoxifen-treated research subjects. Many of the genes in this constellation are involved in the p53 and TNF signaling pathways and are implicated in cancer proliferation. The absence of the estrogen receptor gene in the constellation increases the confidence that this information complements routinely assayed estrogen receptor levels determined by IHC. The test can be used as a predictor of distant metastasis in tamoxifen-treated breast cancer patients. A key finding is the calculation of a metastasis score for breast cancer that predicts a 3.5-fold difference in risk between the 20 % of women at highest risk and the 20 % of women at lowest risk.

Insight® Dx Breast Cancer Profile (*Clarient Inc*.). This has been clinically validated as a prognostic test for women with early-stage, hormone receptor-positive breast cancer. It combines three traditional pathology staging risk factors—tumor size, tumor grade, and lymph node status—with seven key molecular biomarkers, which include ER, PR, HER2, EGFR, bCL2, p53, and myc. The information is then combined with a proprietary algorithm to produce a risk score that assists pathologists and oncologists in clinical decision-making. Clarient conducted an independent study using a set of breast cancer patients from the Royal Perth Hospital in Western Australia to clinically validate the Clarient Insight Dx Breast Cancer Profile. The algorithm demonstrated an accurate, actionable risk recurrence score. In the study, high- and low-risk patients were identified using the Clarient Insight Dx Breast Cancer Profile. The low-risk group had only a 3 % recurrence rate 10

years after surgery. This is equivalent to a negative predictive value of about 97 %, and the corresponding positive predictive value was 39 %. Further details can be seen on the following web site: http://www.clarientinc.com/insightdx.

TaqMan noncoding RNA assays (*Life Technologies*). These assays have helped to uncover regulatory roles of noncoding RNAs in breast cancer. Long intervening noncoding RNAs (lincRNAs) in the HOX loci become systematically dysregulated during breast cancer progression. The lincRNA termed HOTAIR is increased in expression in primary breast tumors and metastases, and HOTAIR expression level in primary tumors is a powerful predictor of eventual metastasis and death (Gupta et al. 2010). Enforced expression of HOTAIR in epithelial cancer cells induced genome-wide retargeting of Polycomb repressive complex 2 (PRC2) to an occupancy pattern more resembling embryonic fibroblasts, leading to altered histone H3 lysine 27 methylation, gene expression, and increased cancer invasiveness and metastasis in a manner dependent on PRC2. Conversely, the loss of HOTAIR can inhibit cancer invasiveness, particularly in cells that possess excessive PRC2 activity. TaqMan noncoding RNA assays can accurately measure expression levels of this molecular biomarker in different breast cancer samples.

MetaStat™ Breast Cancer Test. Scientists at MetaStat Inc. have discovered the microanatomical site in breast cancer by direct visual observation, the MetaSite, the window in the blood vessels through which the metastatic cells squeeze through to enter the bloodstream to begin their deadly journey. The number of these "windows" correlated to the probability of distant site metastases. MetaStat™ Breast Cancer Test uses conventional staining techniques to count these windows, and the count correlates to the risk of metastasis. In clinical trials, the high-risk cohort proved to be 22 times as likely to experience metastasis as the low. The test is inexpensive and fast because archived human tissue samples are used accompanied by their corresponding medical records. The predictions are compared to known outcomes in the corresponding medical records.

16.21.2.13 Racial Factors in the Management of Breast Cancer

Gene expression analysis has identified several breast cancer subtypes, including basal-like, human epidermal growth factor receptor-2 positive/estrogen receptor negative ($HER2^+/ER^-$), luminal A, and luminal B. The basal-like breast cancer subtype was more prevalent among premenopausal African American women (39 %) compared with postmenopausal African American women (14 %) and non-African American women (16 %) of any age (Carey et al. 2006). Although breast cancer is less common in blacks than whites, when black women do develop the disease, they are more likely to die from it, especially if they are under 50. Among those younger women, the breast cancer death rate in blacks is 11 per 100,000, compared with only 6.3 in whites. A higher prevalence of basal-like breast tumors and a lower prevalence of luminal A tumors could contribute to the poor prognosis of young African American women with breast cancer. The finding has no immediate effect on treatment, because there is no treatment that specifically concentrates on basal-like

cancer. Basal-like tumors tend to grow fast and spread quickly, and they are more likely than other types to be fatal. They are not estrogen-dependent and cannot be treated or prevented with estrogen-blocking drugs like tamoxifen or raloxifene. Herceptin, another breast cancer drug, is also useless against these tumors. But efforts are being made to create drugs that will zero in on it. The work involves finding drugs to block specific molecules that these tumors need to grow.

16.21.2.14 RATHER Consortium to Study Personalized Approach to Breast Cancer

RATHER consortium (Rational Therapy for Breast Cancer: Individualized Treatment for Difficult-to-Treat Breast Cancer Subtypes) investigators in Europe received a grant from the EU in 2011 for a 5-year project to study two breast cancer types that are difficult to treat and amount to one-quarter of all breast cancer cases: invasive lobular carcinoma and TNBC. The aim is to profile tumors in search of genetic mutations or other anomalous molecular processes involving kinases in these two cancer types in the hope that these studies will result in differences that are at the root of these cancers and could be targeted by novel drugs. RATHER partners include University College Dublin, Agendia, Oncomark, the Netherlands Cancer Institute, the University of Cambridge, the Curie Institute, Vall d'Hebron Institute of Technology, and Lund University. The partners plan to harmonize their methods to make comparisons between findings from each organization and have started a project database that will be made available to the public. The partners also are working in collaboration with the European Bioinformatics Institute through shared participation in the EurocanPlatform, which is also funded from the EU and will create a resource for use by the European scientific community.

16.21.2.15 TAILORx (Trial Assigning Individualized Options for Treatment)

Hormone therapy alone is usually given to women at low risk for recurrence of breast cancer and chemotherapy followed by hormonal therapy to women at a high risk for recurrence, but there is uncertainty about the best way to handle cases that fall between low and high risk. There is a need for a method of tailoring follow-up treatment that addresses the specific characteristics of a patient's tumor to enable an accurate prediction of what medical treatments will be most effective for long-term alleviation of the disease.

Researchers at the University of Michigan Comprehensive Cancer Center (Ann Arbor, MI) are leading a new study designed to examine whether women with early-stage lymph node-negative breast cancer can be assigned to individualized treatment plans based on certain genes that may predict whether their cancer will recur. The TAILORx study is sponsored by the NCI and will be conducted by all of the NCI-sponsored clinical trial groups that perform breast cancer research studies.

TAILORx seeks to identify women who would not benefit from chemotherapy in order to spare them unnecessary treatment. The study will enroll more than 10,000 women from 900 sites in the USA and Canada. Women recently diagnosed with estrogen receptor-positive, Her2/neu-negative breast cancer, which has not yet spread to the lymph nodes, are eligible for the study. Using Oncotype DX™ (panel of 21 genes with known links to breast cancer), a modern diagnostic test developed by Genomic Health Inc. in collaboration with the National Surgical Adjuvant Breast and Bowel Project, a network of cancer research professionals, TAILORx will determine the most effective cancer treatment, with the fewest side effects, for women with early-stage breast cancer. TAILORx is the first trial to be launched as part of a new NCI program—the Program for the Assessment of Clinical Cancer Tests (PACCT)—which seeks to individualize cancer treatment by using, evaluating, and improving the latest diagnostic tests.

One TAILORx phase III clinical trial at the University of Cincinnati in Ohio uses genetic tests to obtain an individualized and quantitative analysis of how likely a specific patient's breast cancer is to recur. When a patient enrolls in the trial, a tumor tissue sample is sent to a central processing laboratory for Oncotype DX™ analysis. Using a statistical risk prediction model, a score is calculated that represents the specific patient's risk for breast cancer recurrence. The score is determined from the gene expression results using a range of 0–100. Scores between 11 and 25 are considered to be in the intermediate or unclear risk category this trial focuses on. The information gathered from the genetic breast cancer test could give physicians a better understanding of the specific characteristics of their patients' breast tumors, which is critical in planning accurate treatment plans and follow-up.

16.21.2.16 Tamoxifen Therapy for ER-Positive Breast Cancer

For women with ER-positive early breast cancer, treatment with tamoxifen for 5 years substantially reduces the breast cancer mortality rate throughout the first 15 years after diagnosis. In the worldwide Adjuvant Tamoxifen: Longer Against Shorter (ATLAS) trial, women with early breast cancer who had completed 5 years of treatment with tamoxifen were randomly allocated to continue tamoxifen to 10 years or stop at 5 years in the control group (Davies et al. 2013). The results showed that for women with ER-positive disease, continuing tamoxifen to 10 years rather than stopping at 5 years produces a further reduction in recurrence and mortality, particularly after 10 years. These results, taken together with results from previous trials of 5 years of tamoxifen treatment versus none, suggest that 10 years of tamoxifen treatment can reduce breast cancer mortality during the second decade after diagnosis.

16.21.2.17 Triple-Negative Breast Cancer

Subtypes of breast cancer are generally diagnosed based upon the presence or lack of 3 receptors that are known to fuel most breast cancers: progesterone receptors

(PRs), estrogen receptor (ER), and HER2. The most successful treatments for breast cancer target these receptors. Unfortunately, none of these receptors are found in women with TNBC, i.e., the offending tumor is ER-negative, PR-negative, and HER2-negative. Therefore, TNBCs generally do not respond to receptor-targeted treatments. However, this type of breast cancer is typically responsive to chemotherapy. Depending on the stage of its diagnosis, TNBC can be particularly aggressive and more likely to recur than other subtypes of breast cancer. Metastatic TNBC (mTNBC) has a poor prognosis with median survival of 1 year as 30 % of patients suffer a recurrence after first-line treatment. Causative BRCA1 mutations were detected in 9 % of TNBC patients, including patients without significant family histories and/or diagnosed at a later age (Rummel et al. 2013). The mutation frequency in patients <60 years was 11.2–18.3 % in those patients with significant risk factors and 4.6 % in those without, while in patients >60 years, the mutation frequency was 3.5–7.7 % in patients with risk factors and 2.3 % in those without. Thus, the evaluation of additional risk factors in both patients younger and older than 60 years should improve the identification of TNBC patients benefiting from genetic testing of BRCA1.

Whole-genome sequencing (WGS) has revealed previously unreported mutations in mTNBC. Somatic genomic alterations in these advanced tumors, particularly those that might guide targeted therapies, have been cataloged following initial analyses of WGS and transcriptome sequencing data from prospective metastatic mTNBC (Craig et al. 2012). In a sample of 14 tumors from ethnically diverse mTNBC patients, the researchers found significant mutations and other changes in more than a dozen genes through WGS performed on Life Technologies' SOLiD™ 4.0. The most frequently mutated gene among the tumors (7 of 14) was the TP53 tumor suppressor, and aberrations were observed in additional tumor suppressor genes including CTNNA1, which was detected in two of six African American patients (who typically have more aggressive and treatment-resistant disease). Alterations were also seen in the ERBB4 gene, known to be involved in mammary-gland maturation during pregnancy and lactation, but not previously linked to mTNBC. RNA sequencing revealed consistent overexpression of the FOXM1 gene, when tumor gene expression was compared to nonmalignant breast samples. Using an outlier analysis of gene expression comparing one cancer to all the others, the authors detected expression patterns unique to each patient's tumor. Integrative DNA/RNA analysis provided evidence for deregulation of mutated genes. Finally, molecular alterations in several cancers supported targeted therapeutic intervention on clinical trials with known inhibitors, particularly for alterations in the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways. In conclusion, whole genome and transcriptome profiling of mTNBC have provided insights into somatic events occurring in this difficult-to-treat cancer. These genomic data have guided patients to investigational treatment trials and provide hypotheses for future trials in this irremediable cancer. Genome sequencing will eventually become a standard tool for oncologists, enabling them to tailor therapies to the unique genetic profiles of each of their patients.

16.21.2.18 Trends and Future Prospects of Breast Cancer Research

Currently expression profiling can uncover pathway regulation of gene expression and define molecular classes on the basis of integration of the total signals experienced by the cancer cell. The future trends that will have a great impact on breast cancer research are as follows (Miller and Liu 2007):

- The data content will increase. Inclusion of microRNAs (miRNAs) that are not well covered by the existing array technologies would result in greater precision and comprehensiveness.
- The analytical systems will become more informative.
- Metadata sets will emerge that will markedly expand the ability to validate and to model transcriptional networks of biological and clinical significance. This is already taking place with Oncomine and follows the success of other genomic databases. In molecular epidemiology, whole-genome single nucleotide polymorphism (SNP) databases with linked clinical data are being made available to qualified researchers for analysis and data mining.

Primary breast cancers have been analyzed by genomic DNA copy number arrays, DNA methylation, exome sequencing, mRNA arrays, miRNA sequencing, and RPPAs (Koboldt et al. 2012). Integration of information across platforms provided key insights into previously defined gene expression subtypes and demonstrated the existence of four main breast cancer classes when combining data from five platforms, each of which shows significant molecular heterogeneity. Somatic mutations in only three genes (TP53, PIK3CA, and GATA3) occurred at >10 % incidence across all breast cancers; however, there were numerous subtype-associated and novel gene mutations including the enrichment of specific mutations in GATA3, PIK3CA, and MAP3K1 with the luminal A subtype. Two novel protein expression-defined subgroups were identified, possibly produced by stromal/microenvironmental elements, and integrated analyses identified specific signaling pathways dominant in each molecular subtype including an HER2/phosphorylated HER2/EGFR/phosphorylated EGFR signature within the HER2-enriched expression subtype. Comparison of basal-like breast tumors with high-grade serous ovarian tumors showed many molecular features common to both cancers, indicating a related etiology and similar therapeutic opportunities. The biological finding of the four main breast cancer subtypes caused by different subsets of genetic and epigenetic abnormalities leads to the hypothesis that much of the clinically observable plasticity and heterogeneity occurs within, and not across, these major biological subtypes of breast cancer. This is the road map for how breast cancer might be cured in the future. Even within the four major types of breast cancer, individual tumors appear to be driven by their own sets of genetic changes. A wide variety of drugs will most likely need to be developed to be specifically effective for individual tumors. For example, PARP inhibitors that seem to be effective against ovarian cancer may also be tried in basal-like breast cancer, which are most prevalent in younger women, in African Americans, and in women with breast cancer genes BRCA1 and

BRCA2. Two other types of breast cancer, accounting for most cases of the disease, arise from the luminal cells that line milk ducts. These cancers have proteins on their surfaces that grab estrogen, fueling their growth. Genetic analysis divided these cancers into two distinct types. Patients with luminal A cancer had good prognoses while those with luminal B did not, suggesting that perhaps patients with the first kind of tumor might do well with just hormonal therapy to block estrogen from spurring their cancers while those with the second kind might do better with chemotherapy in addition to hormonal therapy. In some cases, genetic aberrations were so strongly associated with one or the other luminal subtype that they appeared to be the actual cause of the cancer. After basal-like cancers, and luminal A and B cancers, the fourth type of breast cancer frequently has extra copies of HER2 gene that drives their growth. Herceptin can block the gene and has changed the prognosis for these patients from one of the worst in breast cancer to one of the best. Although Herceptin is approved for HER2-positive breast cancer patients, the new analysis finds that not all of these tumors are alike in responding to it. This is being investigated in further clinical trials.

This study demonstrates the benefits of integrating genomic and proteomic data, particularly phosphoproteomics, which provided information beyond what the gene expression could. Proteomic data suggests the existence of two distinct phosphoproteomic-based subtypes within the larger gene expression-based HER2 subtype—one exhibiting high HER2 and HER1 signaling activity and the other exhibiting lower levels of such activity. The other example where the protein made one think was the analysis of PI3-kinase signaling, in which a disconnect was found between the PI3K signaling data obtained via the RPPA analysis and their PI3K mutation data. A pathway-based analysis of the PI3K signaling pathway revealed that what are believed to be protein and phosphoproteomic signatures of PI3K activation did not correlate with PI3K mutations, but did correlate with the loss of negative regulators of that pathway, like loss of INPP4B or loss of PTEN. Thus there is a discrepancy between the information from mutation and phosphoproteomics. The challenge now is to figure out which of these many different genetic events or protein signatures are going to be biomarkers of responsiveness to drugs like PI3K or mTOR inhibitors. This work is ongoing and the researchers are currently reanalyzing the genetic data based upon protein and phosphoproteomic endpoints.

16.21.2.19 Understanding Tumor Diversity in Mouse Mammary Cancer Model

Using a finding that the genetic complexity of tumors in mice parallels that in humans, researchers at the Duke University Institute for Genome Sciences and Policy and Duke University Medical Center are starting trial studies in mice, just like human clinical trials, to evaluate whether understanding tumor diversity can improve cancer treatment. Analysis of tumors arising in the MMTV—myc model of mammary carcinogenesis reveals substantial heterogeneity, seen in both histological and expression phenotypes (Andrechek et al. 2009). One of the

MMTV—myc mammary tumor subgroups exhibits metastatic capacity and that the signature derived from the subgroup can predict metastatic potential of human breast cancer. Together, these data reveal that a combination of histological and genomic analyses can uncover substantial heterogeneity in mammary tumor formation and therefore highlight the aspects of tumor phenotype not evident in the population as a whole.

16.21.3 Personalized Management of Ovarian Cancer

A common treatment regimen for ovarian carcinoma consists of tumor debulking, followed by administration of PBC and taxane-based chemotherapy. Due to presentation of disease at advanced stages and development of resistance to therapy, the 5-year survival rate is <40 %. Early diagnosis and identification of nonresponders and patients with primary platinum resistance are important steps toward achieving greater life expectancy for epithelial ovarian cancer (EOC) patients. Gene expression profiles have been established that are associated with overall survival and response to platinum therapy. Despite those encouraging developments, no biomarkers for prediction of response to therapy are yet in clinical use. New approaches for early diagnosis as well as treatment are, therefore, required to improve outcome in this disease. Molecular diagnosis of ovarian cancer was discussed in Chap. 6 as well as in Chap. 5 (Biomarkers).

16.21.3.1 Determining Response to Chemotherapy in Ovarian Cancer

Scientists at the NIH have developed a gene expression profile that predicts ovarian cancer patient response to chemotherapy. Two gene signatures are available for licensing. One gene signature can predict whether a patient will initially respond to standard platinum–paclitaxel chemotherapy but will relapse within 6 months of completing treatment. A second gene signature identifies patients who will show no response to therapy. This method may enable clinicians to identify patients who may be candidates for additional and/or novel chemotherapy drugs and effectively choose appropriate cancer treatment. A unique feature of this signature is its derivation from pure, microdissected isolates of ovarian tumor cells, rather than undissected tissue. An advantage of this approach is that the resulting gene list is specific to the cell type which causes the disease.

A variant in the 3′UTR of the K-ras oncogene, referred to as the K-ras variant, is associated with both cancer risk and altered tumor biology. K-ras variant can act as a biomarker of outcome in EOC. A study has shown that postmenopausal EOC patients with the K-ras variant are significantly more likely to die of ovarian cancer by multivariate analysis and are significantly more likely to be platinum resistant (Ratner et al. 2012). In addition, direct targeting of the K-ras variant leads to a significant reduction in EOC cell growth and survival in vitro. These findings confirm

the importance of the K-ras variant in EOC and indicate that the K-ras variant is a biomarker of poor outcome in EOC likely due to platinum resistance. In addition, this study supports the hypothesis that these tumors have continued dependence on such 3′UTR lesions and that direct targeting may be a viable future treatment approach.

16.21.3.2 Prognosis of Ovarian Cancer Based on CLOVAR

The Cancer Genome Atlas catalog has been used to develop a prognostic model of HGS-OvCa classification called "Classification of Ovarian Cancer" (CLOVAR), which is based on subtype and survival gene expression profiles (Verhaak et al. 2013). Rather than relying on the 193 genes previously used in the signature of survival, the researchers focused on a smaller set, choosing the 100 genes whose expression was the most or least indicative of good prognosis from the full 481 TCGA HGS-OvCa sample set. Similarly, the researchers then revamped the subtype gene expression signature by narrowing the initial list of 800 genes down to 100 genes. Applied to a validation set, this new survival signature could stratify HGS-OvCa samples into a good-prognosis group and a poor-prognosis group. The worst outcome group, accounting for 23 % of all cases, was associated with a median survival of 23 months and a platinum resistance rate of 63 % versus a median survival of 46 months and platinum resistance rate of 23 % in other cases. Associating the outcome prediction model with BRCA1/BRCA2 mutation status, residual disease after surgery, and disease stage further optimized outcome classification. The results of this study suggest that combining CLOVAR survival, immunoreactive, and mesenchymal scores with BRCA1/BRCA2 mutation status provides optimal outcome predictions. The spectrum of outcomes observed in this study and their association with CLOVAR signatures suggests variations in underlying tumor biology. The association uncovered between the CLOVAR survival, immunoreactive, and mesenchymal gene signature scores suggests an active role for the stromal tumor microenvironment in the pathogenesis of HGS-OvCa and may indicate possible targets for cancer therapies. An improved understanding of ovarian carcinoma development may ultimately lead to more effective treatments. Prospective validation of the CLOVAR model in the context of additional prognostic variables such as BRCA mutation status, age, grade, and residual disease may provide a rationale for optimal combination of patient and treatment regimens. A prospective study would be most revealing when assessing the predictive capacities of the CLOVAR signatures in conjunction with other prognostic factors.

16.21.3.3 Recurrent and Drug-Resistant Ovarian Cancer

To identify the best treatment for recurrent ovarian cancer, researchers at Yale School of Medicine (Harford, CT) are studying a technology called the Yale apoptosis assay in combination with another technology called the ChemoFX assay,

which could double the response rate to existing drugs. In patients with recurrent ovarian cancer, it is often difficult to select an effective treatment because the tumor develops resistance to many drugs. Currently, physicians select a drug and must wait about 6 months to see whether it is effective on a particular patient. These two new assays will take the guesswork out of cancer treatment. Yale apoptosis assay is based on a biological principle that when a drug is effective, it will induce apoptosis in the cancer cell. If the cancer cell is resistant to a drug, apoptosis does not occur. The ChemoFX assay will determine whether a drug stops tumor growth. Used together, both assays will distinguish drugs that can stop the growth of the tumor and/or kill the tumor. This was not possible before. The technology will be studied with various cancers, starting with ovarian cancer. Each assay will be evaluated independently and then in combination in a multicenter clinical trial. The Yale research team partnered with PTI developers of the ChemoFX assay. PTI exclusively licensed the Yale apoptosis assay from Yale. A study in 2009 at Duke University showed that over 50 % of physicians followed results of ChemoFx® in management of ovarian cancer and results changed physician behavior. The use of ChemoFx® results in cost savings of $2,900–8,100 per patient per round for primary or recurrent ovarian cancer cases over a six-cycle treatment period.

The high incidence of recurrence attributable to MDR and the multiple histologic phenotypes indicative of multipotency suggests a stem cell-like etiology of ovarian cancer. A side population (SP) cell has been identified and characterized from two distinct genetically engineered mouse ovarian cancer cell lines (Szotek et al. 2006). Differential efflux of a DNA-binding dye from these cell lines defined the human breast cancer-resistance protein 1-expressing, verapamil-sensitive SP of candidate CSCs. In vivo, mouse SP cells formed measurable tumors sooner than non-SP (NSP) cells when equal numbers were injected into the dorsal fat pad of nude mice. The presence of Mullerian-inhibiting substance (MIS) signaling pathway transduction molecules in both SP and NSP mouse cells led us to investigate the efficacy of MIS against these populations in comparison with traditional chemotherapies. MIS inhibited the proliferation of both SP and NSP cells, whereas the lipophilic chemotherapeutic agent doxorubicin more significantly inhibited the NSP cells. Finally, breast cancer-resistance protein 1-expressing verapamil-sensitive SPs were identified in human ovarian cancer cell lines and primary ascites cells from patients with ovarian cancer. In the future, individualized therapy must incorporate analysis of the stem cell-like subpopulation of ovarian cancer cells when designing therapeutic strategies for ovarian cancer patients.

High-grade serous cancer (HGSC), the most common subtype of ovarian cancer, often becomes resistant to chemotherapy, leading to poor patient outcomes. Intratumor heterogeneity occurs in nearly all solid cancers, including ovarian cancer, contributing to the development of resistance mechanisms. Past studies have identified a handful of resistance-related mutations in ovarian cancer, but unidentified copy number variations (CNVs) could contribute to this process as well. A study has examined the spatial and temporal genomic variation in HGSC using high-resolution Affymetrix SNP 6.0 arrays (Cowin et al. 2012). Multiple metastatic lesions from individual patients were analyzed along with 22 paired pretreatment and

posttreatment samples. The authors documented regions of differential DNA copy number between multiple tumor biopsies that correlated with altered expression of genes involved in cell polarity and adhesion. In the paired primary and relapse cohort, they observed a greater degree of genomic change in tumors from patients that were initially sensitive to chemotherapy and had longer progression-free interval compared with tumors from patients that were resistant to primary chemotherapy. Notably, deletion or downregulation of the lipid transporter LRP1B emerged as a significant correlate of acquired resistance in the analysis. Functional studies showed that reducing LRP1B expression was sufficient to reduce the sensitivity of HGSC cell lines to liposomal doxorubicin, but not to doxorubicin, whereas LRP1B overexpression was sufficient to increase sensitivity to liposomal doxorubicin. Together, these findings underscore the large degree of variation in DNA copy number in spatially and temporally separated tumors in HGSC patients. LRP1B is defined as a potential contributor to the emergence of chemotherapy resistance in these patients and may serve as a biomarker for such acquired resistance. Mapping the mechanisms that confer resistance may enable prediction of whether some women are likely to respond to a certain drug or not and find ways of reversing resistance.

Quantitative RT-PCR of 3 miRs (miR-484, miR-642, and miR-217) involved in angiogenesis is able to predict chemoresistance of ovarian cancer (Vecchione et al. 2013). Additional analysis of miR-484 reveals that the sensitive phenotype is caused by a modulation of tumor vasculature through the regulation of the VEGFB and VEGFR2 pathways. These findings suggest that blockage of VEGF by use of an anti-VEGFA antibody may not be sufficient to improve survival in ovarian cancer patients unless VEGFB signaling is also blocked. Alternatively, small compounds, such as functionalized nanoparticles targeting the VEGFR1 and VEGFR2 receptors, could be used as effective therapy in these patients, changing the course of prognosis and treatment of ovarian cancer (Liu et al. 2011).

16.21.3.4 Pathway-Targeted Therapies for Ovarian Cancer

Mouse ovarian epithelial tumor cell lines that contain various combinations of genetic alterations in the p53, c-myc, K-ras, and Akt genes have been used as model for the molecular characterization of pathway-targeted therapy. Response to a particular anticancer drug can be related to the signaling pathway involved. Effect of rapamycin on cell proliferation, tumor growth, and the accumulation of peritoneal ascites were investigated in this model using both in vitro and in vivo approaches (Xing and Orsulic 2005). Rapamycin effectively inhibits the growth of tumors that rely on Akt signaling for proliferation, whereas tumors in which Akt signaling is not the driving force in proliferation are resistant to rapamycin. The introduction of activated Akt to the rapamycin-resistant cells does not render the cells susceptible to rapamycin if they can use alternative pathways for survival and proliferation. Therefore, the rapamycin-sensitive tumors develop resistance to rapamycin when presented with alternative survival pathways, such as the mitogen-activated

extracellular kinase signaling pathway. The combination of rapamycin and the mitogen-activated extracellular kinase inhibitor PD98059 is required to diminish proliferation in these cell lines. These results indicate that mTOR inhibitors may be effective in a subset of tumors that depend on Akt activity for survival but not effective in all tumors that exhibit Akt activation. Tumors with alternative survival pathways may require the inactivation of multiple individual pathways for successful treatment. These results have significant implications for the use of pathway-targeted therapy in advanced human ovarian cancers, which typically display numerous genetic alterations that are likely to require impairment of multiple molecular pathways for successful treatment. Interruption of multiple specific biochemical pathways may be a promising therapeutic strategy in ovarian carcinomas that exhibit resistance to an individual targeted therapy. This strategy may be useful for developing personalized therapies for ovarian cancer.

Human ovarian CSCs have been characterized and shown to have a distinctive genetic profile that confers them with the capacity to recapitulate the original tumor, proliferate with chemotherapy, and promote recurrence (Alvero et al. 2009). CSCs identified in ovarian cancer cells isolated from ascites and solid tumors are characterized by cytokine and chemokine production, high capacity for repair, chemoresistance to conventional chemotherapies, and resistance to TNFα-mediated apoptosis. Chemotherapy eliminates the bulk of the tumor, but it leaves a core of CSCs with high capacity for repair and renewal. The molecular properties identified in these cells may explain some of the unique characteristics of CSCs that control self-renewal and drive metastasis. The identification and cloning of human ovarian CSCs can aid in the development of better therapeutic approaches for ovarian cancer patients.

Resistance to platinum therapy is a major obstacle that needs to be overcome in the treatment of ovarian cancer patients. The high rates and patterns of therapeutic failure seen in patients are consistent with a steady accumulation of drug-resistant CSCs. A study has demonstrated that the notch signaling pathway and notch3 in particular are critical for the regulation of CSCs and tumor resistance to platinum (McAuliffe et al. 2012). Notch3 overexpression in tumor cells results in expansion of CSCs and increased platinum chemoresistance. In contrast, γ-secretase inhibitor (GSI), a notch pathway inhibitor, depletes CSCs and increases tumor sensitivity to platinum. Similarly, a notch3 siRNA knockdown increases the response to platinum therapy, further demonstrating that modulation of tumor chemosensitivity by GSI is notch-specific. Most importantly, the cisplatin/GSI combination is the only treatment that effectively eliminates both CSCs and the bulk of tumor cells, indicating that a dual combination targeting both populations is needed for tumor eradication. In addition, cisplatin/GSI combination therapy has a synergistic cytotoxic effect in notch-dependent tumor cells by enhancing the DNA damage response, G2/M cell cycle arrest, and apoptosis. These findings indicate that targeting the Notch pathway significantly increases tumor sensitivity to platinum therapy. Both platinum-resistant and platinum-sensitive relapses may benefit from such an approach as clinical data suggest that all relapses after platinum therapy are increasingly platinum resistant.

Two strategies for targeted therapy have emerged with promising results: PARP enzyme inhibitors and targeting angiogenesis, but the development of a convenient and accurate method to identify patients likely to benefit from these remains a challenge (Gómez-Raposo et al. 2011).

A catalog of molecular aberrations that cause ovarian cancer is critical for developing and deploying therapies that will improve patients' lives. The Cancer Genome Atlas project has analyzed mRNA expression, miRNA expression, promoter methylation, and DNA copy number in high-grade serous ovarian adenocarcinomas and the DNA sequences of exons from coding genes in most of these tumors (The Cancer Genome Atlas Research Network 2011). The equipment used included Agilent, Illumina, and Affymetrix arrays to look at CNV, mRNA expression, miRNA expression, and methylation profiles of tumor samples. Whole-exome sequencing was carried out on a subset of these. High-grade serous ovarian cancer was found to be characterized by TP53 mutations in almost all tumors. Pathway analyses suggested that homologous recombination was defective in about half of the tumors analyzed and that notch and FOXM1 signaling are involved in serous ovarian cancer pathophysiology. Although relatively few genes were found to contain recurrent mutations in the ovarian cancer, the researchers tracked down numerous CNVs and several frequently mutated pathways, along with miRNA, methylation, and transcription signatures that hold promise for categorizing ovarian cancer and predicting survival outcomes. Overall, these discoveries set the stage for approaches to the treatment of high-grade serous ovarian cancer in which aberrant genes or networks are detected and targeted with therapies selected to be effective against these specific aberrations.

16.21.4 Personalized Management of Head and Neck Cancer

Molecular-targeted therapy in head and neck squamous cell carcinoma (HNSCC) continues to make strides and holds much promise. Cetuximab remains the sole FDA-approved molecular-targeted therapy available for HNSCC, though several new biologic agents targeting the EGFR and other pathways are currently in the regulatory approval pipeline. While targeted therapies have the potential to be personalized, their current use in HNSCC is not personalized. This is illustrated for EGFR-targeted drugs, where EGFR as a molecular target has yet to be individualized for HNSCC. Future research needs to identify factors that correlate with response (or lack of one) and the underlying genotype–phenotype relationship that dictates this response. Comprehensive exploration of genetic and epigenetic landscapes in HNSCC is opening new frontiers to further enlighten and mechanistically inform newer as well as existing molecular targets and to set a course for eventually translating these discoveries into therapies for patients. A snapshot of the evolution of molecular subtyping in HNSCC and its current clinical applicability, as well as new emergent paradigms with implications for controlling this disease in the future, has been presented in an opinion article (Worsham et al. 2012).

16.21.4.1 Relevance of Biomarkers of HPV-Related Head and Neck Cancer

Some reports have associated a subset of HNSCC with high-risk human papillomaviruses (HPVs), particularly HPV16, the same subset of HPVs responsible for the majority of cervical and anogenital cancers. A positive test for HPV DNA alone was not significantly linked to HNSCC outcomes. On the other hand, when found in combination with E6 and E7 expression, a positive HPV16 test did coincide with improved oropharyngeal cancer outcomes. Likewise, elevated levels of p16 in a tumor were not especially informative on their own, though they did correspond to better oropharyngeal cancer survival when found together with positive blood tests for E6 and E7. Based on these findings, it is concluded that a stronger association of HPV presence with prognosis (assessed by all-cause survival) is observed when HPV-associated HNSCC is defined using tumor status (HPV DNA or P16) and HPV E6/E7 serology in combination rather than using tumor HPV status alone (Liang et al. 2012).

Another study on oropharyngeal squamous cell carcinomas (OPSCC) found its own evidence arguing against the use of HPV DNA as a solo biomarker for HPV-associated cancer (Holzinger et al. 2012). They tested OPSCC tumors for HPV DNA and p16. They also considered the viral load in the tumors and looked for gene expression profiles resembling those described in cervical carcinoma, another cancer associated with HPV infection. Again, the presence of HPV DNA appeared to be a poor indicator of HPV-associated cancers or predictor of cancer outcomes. Whereas nearly half of the tumors tested positive for HPV16 DNA, just 16 and 20 % had high viral loads and cervical cancer-like expression profiles, respectively. The researchers found that a subset of HPV DNA-positive tumors with high viral load or HPV-associated expression patterns belonged to individuals with better outcomes. In particular, they found that cervical cancer-like expression profiles in OPSCC tumors coincided with the most favorable outcomes, while high viral load in the tumors came a close second. Once standardized assays for these biomarkers, applicable in routine clinical laboratories, are established, they will allow precise identification of patients with oropharyngeal cancer with or without HPV-driven cancers and, thus, will influence prognosis and potentially treatment decisions. More research is needed to understand whether the patterns described in the new studies hold in other populations and to tease apart the prognostic importance of HPV infection in relation to additional prognostic biomarkers.

16.21.5 Personalized Management of Hematological Malignancies

Considerable work has been done on molecular cytogenetics of hematological malignancies and a number of diagnostics and therapies are available or under development. Myeloproliferative disorders include several pathologies sharing the common feature of being clonal hematopoietic stem cell diseases. The molecular basis of CML was characterized many years ago with the discovery of the t(9;22) translocation and its product the BCR–ABL oncoprotein. The finding of a recurrent

mutation in the Janus 2 tyrosine kinase (JAK2) gene was a major advance in understanding of the pathogenesis of several other myeloproliferative disorders, including polycythemia vera, essential thrombocythemia, and idiopathic myelofibrosis. Such a recurrent and unique mutation leading to a tyrosine kinase deregulation would make a suitable target for the development of specific therapies. QIAGEN Marseille has worldwide exclusive intellectual property rights to a test based on mutations in the JAK2 gene.

16.21.5.1 Personalized Management of Acute Lymphoblastic Leukemia

Progress in the molecular classification of ALL with the use of DNA microarrays combined with methods to assess the functional significance of newly discovered genes or through proteomic techniques will lead to the identification of targets for specific treatments. Imatinib mesylate, introduced for the treatment of BCR–ABL-positive CML, inhibits the BCR–ABL fusion protein and other constitutively active tyrosine kinases and induces transient remissions of BCR–ABL-positive ALL as well as partial responses in other cancers and is the forerunner of a new generation of molecularly targeted anticancer drugs. Other potentially useful agents that are under development include inhibitors of FLT3 tyrosine kinases for use against leukemias characterized by activating mutations of this kinase and inhibitors of histone deacetylase for leukemias such as TEL–AML1-positive ALL. Further refinements in the molecular classification of ALL, together with the identification of genetic features that affect the efficacy and toxicity of antileukemic therapy, will provide unique opportunities to devise treatment plans for individual patients and thus to realize the elusive goal of cure in all patients, regardless of their presenting characteristics. ALL is treated with a cocktail of chemotherapeutic agents that include 6-mercaptopurine, 6-thioguanine, and azathiopurine. These drugs are broken down by the TPMT. Those lacking functional TPMT can suffer severe toxicity or death, but these patients can be treated with doses that are much lower than the standard regimen. Physicians at St. Jude Children's Hospital (Memphis, TN) and at the Mayo Clinic (Rochester, MN) are prescreening patients to determine if they have functional or nonfunctional enzyme TPMT. The dosage of the components in the chemotherapeutic cocktail is then tailored precisely to the patient's molecular makeup—personalized prescribing. TPMT genotype also has a substantial impact on MRD after administration of mercaptopurine in the early course of childhood ALL, most likely through modulation of mercaptopurine dose intensity (Stanulla et al. 2005). These findings support a role for MRD analyses in the assessment of genotype–phenotype associations in multiagent chemotherapeutic trials. Investigators at St. Jude Children's Research Hospital have also developed a relatively simple and inexpensive test that identifies children with ALL who have responded well enough to their first round of chemotherapy that they might be successfully treated with a much less aggressive follow-up treatment.

Genetic variation in the enzymes of the folic acid cycle, one-carbon transfer, immune surveillance, drug metabolism, and transport may determine some of the

variability in treatment response of ALL patients. Despite recent advances in this area, further work is needed to develop clinically useful genetic predictors of leukemia treatment response (Cunningham and Aplenc 2007).

Risk factors for CNS relapse in childhood ALL included the genetic abnormality t(1;19)(TCF3–PBX1), any CNS involvement at diagnosis, and T-cell immunophenotype. At St. Jude Children's Hospital, personalized therapy is applied based on molecular genetics of ALL, pharmacogenetic traits of patients, and pharmacodynamic principles. The activity of drug-metabolizing enzymes of each patient is determined prospectively and the dosage of chemotherapy is adjusted accordingly. It was demonstrated that with effective risk-adjusted personalized chemotherapy, prophylactic cranial irradiation can be safely omitted from the treatment of childhood ALL (Pui et al. 2009). This chemotherapy approach produced a projected cure rate of 90 % for all the patients, which is the best treatment result reported to date.

16.21.5.2 Personalized Management of Acute Myeloid Leukemia

Two molecular tests for acute myeloid leukemia (AML) from Genzyme Diagnostics are relevant to personalized management: FLT3 Mutation Analysis and WT1 RQ-PCR. FLT3 mutations are considered a prognostic indicator of poor survival and response to standard chemotherapies. Approximately 30 % of patients with AML have FLT3 mutations. WT1 RQ-PCR test is designed to detect MRD or very low levels of disease. The WT1 gene is expressed in approximately 90 % of patients with AML. This test allows physicians to monitor AML patients for early relapse during and following therapy. Both of these tests may enable oncologists to better manage their patients. Laboratory for Personalized Molecular Medicine is developing a companion diagnostic for the identification of FLT3-positive AML patients for the treatment with Novartis' midostaurin, or PKC412, a targeted small-molecule inhibitor of FLT3 tyrosine kinase, which is currently in phase III clinical trials. The American Society of Clinical Oncology and the National Comprehensive Cancer Network recommend testing for the FLT3 mutation and determination of FLT3 status as a standard of care for patients diagnosed with AML.

Activating internal tandem duplication (ITD) mutations in FLT3 (FLT3-ITD) are associated with a poor prognosis. Scientific evidence including the lack of convincing clinical activity of early FLT3 inhibitors suggests that FLT3-ITD probably represents a passenger lesion. Point mutations have been reported at three residues within the kinase domain of FLT3-ITD that confer substantial in vitro resistance in AML patients to AC220 (quizartinib), an active investigational inhibitor of FLT3 (Smith et al. 2012). These findings demonstrate that FLT3-ITD can represent a driver lesion and valid therapeutic target in human AML. AC220-resistant FLT3 kinase domain mutants represent high-value targets for future FLT3 inhibitor development efforts.

Risk stratification in AML is currently based on pretreatment characteristics. It remains to be established whether relapse risk can be better predicted through assessment of MRD. One proposed marker is the Wilms tumor gene WT1, which is overexpressed in most patients with AML, thus providing a putative target for

immunotherapy. An international collaborative study coordinated by the European Leukemia Network (ELN) consortium on standardization of WT1 testing for risk stratification in AML has been published (Cilloni et al. 2009). The objective of this study was to select the best-performing WT1 assay and to assess the value of WT1 monitoring during AML treatment to estimate the risk of relapse. Results of the study show that the high-performance WT1 assay designed by the ELN group is adapted to MRD assessment. This specific assay developed and validated in the context of this study, WT1 ProfileQuant® (QIAGEN Marseille), is CE marked and can be used with most RQ-PCR instruments. Application of a standardized WT1 assay provides independent prognostic information in AML, lending support to incorporation of early assessment of MRD to develop more robust risk scores, to enhance risk stratification, and to identify patients who may benefit from allogeneic transplantation.

Cytarabine (ara-C) is the most effective agent for the treatment of childhood AML, but aberrant expression of enzymes involved in the transport/metabolism of ara-C could explain drug resistance. Human equilibrative nucleoside transporter-1 (hENT1) mRNA expression and ara-C sensitivity were significantly correlated with threefold lower hENT1 mRNA levels in resistant patients (Hubeek et al. 2005). Thus, decreased expression of hENT1, which transports ara-C across the cell membrane, appears to be a major factor in ara-C resistance in childhood AML. In 2011, Clavis Pharma received a Norwegian government grant to develop a flow cytometry method for the detection and quantification of human hENT1 in patients suffering from AML, which will enable the selection of the subpopulation of AML patients who are likely to benefit most from treatment with the novel anticancer drug elacytarabine.

16.21.5.3 Personalized Management of Chronic Lymphocytic Leukemia

CLL is the most common leukemia in the Western world with the majority of cases occurring in patients over the age of 55. It usually progresses slowly and is characterized by the accumulation of lymphocytes, which can overwhelm the bone marrow and invade the bloodstream, eventually spreading to the spleen, liver, and other solid organs. CLL, however, has a highly variable clinical course: slowly progressive in some, whereas others have an aggressive disease. In the last quarter of the twentieth century, prognosis and treatment decisions were based on clinical staging systems. In the twenty-first century, biomarkers have enabled a more refined prognostic stratification. In spite of advancements in WGS, CLL is not associated with a specific genetic abnormality. However, B-cell receptor signaling, which may be constitutively expressed, antigen induced, or both, plays a critical role in driving cell proliferation in CLL and their survival through the cascade of protein kinases.

The elimination of CLL to an extremely low level may improve the overall survival and treatment-free survival. According to a study, 84 % of patients who had no detectable CLL cells after receiving Campath (alemtuzumab) had survived for at least 5 years; 20 % the same patients had previously failed to respond or had relapsed after receiving other chemotherapy for their disease (Moreton et al. 2005). CLL patients who relapse from or are refractory to chemotherapy have the poorest

prognosis with a median survival of 10 months. Availability of a test to detect MRD in patients with B-cell CLL to complement the treatment with Campath® is an example of combining diagnostics with therapy to improve the treatment.

Bruton's tyrosine kinase (BTK) is a downstream target of the B-cell receptor, and a new class of drugs that are capable of inhibiting BTK has been developed. Ibrutinib, an oral inhibitor, has shown activity in a small series of patients with relapsed or refractory CLL or small lymphocytic lymphoma (Advani et al. 2013). In a phase Ib clinical trial, ibrutinib was associated with a high frequency of durable remissions in patients with relapsed or refractory CLL and small lymphocytic lymphoma, including patients with high-risk genetic lesions (Byrd et al. 2013). This is an example of trend in management of hematologic cancers, which is shifting from a chemotherapy-based approach to treatments aimed at mechanisms of disease. New prognostic subgroups in CLL based on integrated mutational and cytogenetic analysis are (Rossi et al. 2013):

1. High risk (10-year survival <30 %): TP53 abnormalities, BIRC3 abnormalities, or both
2. Intermediate risk: notch1 mutations, SF3B1 mutations, or both, with or without 11q22.3 deletion
3. Low risk: trisomy 12 or normal cytogenetic profile
4. Very low risk (10-year survival ~70 %): 13q14 deletion only

16.21.5.4 Personalized Management of Multiple Myeloma

Despite improvements in therapy, the 5-year survival rate in multiple myeloma is only 32 % and durable responses are rare. Multiple myeloma is a neoplasia of clonally expanded malignant bone marrow plasma cells. Previously two genetic subtypes of myeloma were known: (1) hyperdiploid multiple myeloma (MM) characterized by extra copies of entire chromosomes and patients with this subtype appear to fare better, and (2) non-hyperdiploid form lacks these extra chromosomes and instead has abnormal rearrangements between different chromosomes with worse outlook for the patients with this subtype. The roles played by various abnormalities in the initiation and progression of myeloma are only beginning to be understood, but it has been observed that different abnormalities vary from one patient to the other.

Pharmacogenomic studies in multiple myeloma are helping to set the stage for individualized therapy. Although relatively few in numbers, these studies are already providing new therapeutic targets and avenues for drug discoveries as well as contributing to novel prognostic markers in multiple myeloma. Genetics and GEP technology have improved molecular-based patient stratification and prognostic staging, expanded knowledge of the molecular mechanism of chemotherapeutic agents, and provided a better understanding of multiple myeloma.

Distinct genetic subtypes of MM have different prognoses and might be treated most effectively with drugs specifically targeted to those subtypes. For further analysis of many DNA alterations in the MM genome, an algorithm was created based

on a computational method, which was used to group the results in a way that yielded distinctive genomic features from the CGH data. Four distinct myeloma subtypes based on genetic patterns emerged: two of them corresponded to the non-hyperdiploid and hyperdiploid types, but the latter was found to contain two further subdivisions, called k1 and k2. When these subgroups were checked against the records of the patients from whom the samples were taken, it showed that those with the k1 pattern had a longer survival than those with k2. These results define new disease subgroups of MM that can be correlated with different clinical outcomes. The findings pave the way for treatments tailored to a patient's specific form of the disease and also narrow down areas of the chromosomes in myeloma cells likely to contain undiscovered genetic aberrations that drive myeloma, and which might turn out to be vulnerable to targeted designer drugs.

Researchers at Mayo Clinic Cancer Center, in cooperation with industry partners, have identified tumor-specific alterations in the cellular pathway by which the MM drug bortezomib (Velcade) works and they have identified nine new genetic mutations in cancer cells that should increase a patient's chance of responding to the agent. These findings may help physicians tailor treatment to patients with MM. Bortezomib seems to work in about one-third of patients who use it, but up to now it was not possible to predict which ones. Investigators have identified a group that will likely respond because these nine mutations seem to be present in at least 25 % of newly diagnosed patients. Multiple genetic mutations in the other NF-κB pathway, the so-called noncanonical pathway, make the tumor more dependent on that pathway and consequently more susceptible to bortezomib treatment. Identifying these mutations in patients will help the decision as to which patients should be treated with bortezomib, probably as an initial therapy. A test is in development to check for activation of the noncanonical NF-κB pathway in patients. Now that the mutations have been identified, drug designers may be able to fashion new therapies that are more specific to these genetic alterations and, therefore, less toxic. These mutations represent good targets for drug development.

Despite overwhelming genomic chaos in multiple myeloma (MM), expression patterns within tumor samples are remarkably stable and reproducible. Unique expression patterns associated with recurrent chromosomal translocations and ploidy changes defined molecular classes with differing clinical features and outcomes. Combined molecular techniques also dissected two distinct, reproducible forms of hyperdiploid disease and have molecularly defined MM with high risk for poor clinical outcome. GEP is now used to risk-stratify patients with newly diagnosed MM. Groups with high-risk features are evident in all GEP-defined MM classes, and GEP studies of serial samples showed that risk increases over time, with relapsed disease showing dramatic GEP shifts toward a signature of poor outcomes. This suggests a common mechanism of disease evolution and potentially reflects preferential expansion of therapy-resistant cells. Correlating GEP-defined disease class and risk with outcomes of therapeutic regimens reveals class-specific benefits for individual agents, as well as mechanistic insights into drug sensitivity and resistance (Zhou et al. 2009).

Signal Genetics' scientists have analyzed the expression levels of thousands of human genes that are considered to be linked to MM. Clustering analysis of these various genes gave rise to the 70 most relevant myeloma-linked genes used to make up the patient's gene expression profile, which is the basis of My Prognostic Risk Signature™ (MyPRS™) as a diagnostic supplement for MM, which can help to design a personalized regimen. The median survival rate for MM in the USA is 2.5–3 years, but personalized approach can raise the median survival rate to 6–7 years.

16.21.5.5 Personalized Management of Myelodysplastic Syndrome

In myelodysplastic syndrome (MDS), cytogenetic analyses are mandatory for risk stratification and for monitoring response to drug treatment. Low-dose demethylating agents such as 5-aza-2′-deoxycytidine (decitabine) and 5-azacytidine (azacitidine, Vidaza) have been explored for the treatment of MDS aiming to revert a methylator phenotype. Decitabine treatment is associated with a response rate that is higher in patients with high-risk cytogenetics (i.e., complex karyotype and/or abnormalities of chromosome 7) than in patients with intermediate-risk cytogenetics (two abnormalities or single abnormalities excluding 5q−, 20q−, and -Y). Following decitabine treatment of patients with abnormal karyotype, approximately one-third achieve a major cytogenetic response that can be confirmed by FISH analyses, while in two-thirds of patients, the abnormal karyotype persists, but hematologic improvement may be observed during continued treatment. The most frequently studied gene in myelodysplasia is the cell cycle regulator p15. Hypermethylation of p15 in MDS is reversed during treatment with decitabine, resulting in reactivation of this gene.

Somatic mutations in MDS may influence the clinical phenotype but are not included in current prognostic scoring systems. Combination of genomic approaches, including NGS and mass spectrometry-based genotyping, identified somatic mutations in 18 genes in samples of bone marrow aspirate from patients with MDS and associated them with specific clinical features (Bejar et al. 2011). Mutations in TP53, EZH2, ETV6, RUNX1, and ASXL1 were found to be predictors of poor overall survival in MDS patients independently of established risk factors.

16.21.6 Personalized Management of Lymphomas

16.21.6.1 Personalized Management B-Cell Lymphomas

B-cell lymphomas are tumors of cells of the immune system that include Hodgkin and non-Hodgkin lymphomas such as follicular lymphoma. B cells are the immune system cells that produce antibodies. Genetic aberrations can cause B cells to

multiply uncontrollably, causing B-cell lymphomas. A gene called bCL6 codes for a protein, which is a transcriptional repressor, i.e., it can shut off the functioning of genes in B cells and other cells of the immune system and prevent them from being expressed. The bCL6 protein is normally produced only during a specific stage of B-cell development and is never made again. But deregulation of bCL6 can cause the protein to be produced when it should not be. The unwelcome presence of the bCL6 protein blocks the expression of important genes that normally protect cells from becoming cancerous. A peptide called BPI has shown promise in treating B-cell lymphomas by specifically blocking the cancer-causing effects of the bCL6 protein. However, until now, there has been no way to distinguish between diffuse large-B-cell lymphomas that are caused by bCL6 deregulation and those cases in which bCL6 is expressed but does not actually drive the cancer. In an effort to identify cases of lymphoma that are uniquely susceptible to BPI inhibitor therapy, genomic array ChIP-on-chip was used to identify the cohort of direct bCL6 target genes (Polo et al. 2007). In primary diffuse large-B-cell lymphomas classified on the basis of gene expression profiles, these bCL6 target genes were clearly differentially regulated in BCR tumors, a subset of DLBCLs with increased bCL6 expression and more frequent bCL6 translocations. Only BCR tumors were highly sensitive to the bCL6 peptide inhibitor, BPI. This genetic signature can help physicians to enroll patients in clinical trials of the new targeted therapy who are most likely to benefit from it. Patients who do not fit this genetic profile will be spared a drug treatment that would be ineffective.

A combination of targeted sequencing and microarray analyses in non-Hodgkin lymphoma cell lines was used in a study to find mutation and gene expression patterns linked to resistance or sensitivity to dacetuzumab, a CD40-stimulating antibody being investigated for the treatment of diffuse large-B-cell lymphomas and other B-cell cancers (Burington et al. 2011). The results showed that a gene expression signature associated with activation of the TNF CD40 pathway can help predict response to a new B-cell cancer treatment targeting the pathway. A qRT-PCR assay, which assesses the expression of 15 genes, was subsequently used to predict dacetuzumab treatment response in cell lines, mouse xenograft models, and clinical samples. Generally, cell lines harboring mutations in the tumor suppressor gene p53 were more likely to respond to the CD40 stimulation treatment, as were cell lines with widespread DNA damage or unusually high proliferation rates. Elevated levels of the transcriptional repressor proto-oncogene bCL6 also coincided with treatment sensitivity. In contrast, cell lines exhibiting expression patterns consistent with CD40 pathway activation prior to treatment tended to be resistant. In clinical samples from diffuse large-B-cell lymphoma patients treated with dacetuzumab during phase I/II trials of the therapy, the response signature accurately predicted treatment response—specifically, tumor shrinkage—for 80 % of the cases. Patients classified as having treatment-sensitive tumors based on the expression signature also had significantly longer progression-free survival times following dacetuzumab treatment than those with tumors classified as treatment resistant.

16.21.6.2 Personalized Vaccine for Follicular Lymphoma

Follicular lymphoma is considered incurable, although cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy can induce sequential remissions. In one study, patients with follicular lymphoma were vaccinated periodically for more than 2 years with autologous lymphoma-derived idiotype protein vaccine (Inoges et al. 2006). The vaccine presents a tumor protein to the patients in such a way that their immune systems recognize it and destroy any cells bearing that protein. Idiotypic vaccination induced a specific immune response in the majority of patients with follicular lymphoma. Specific immune response was associated with a dramatic and highly statistically significant increase in disease-free survival. This is the first formal demonstration of clinical benefit associated with the use of a human cancer vaccine. Such clinical trials cannot be randomized as each patient serves as his or her own control. A second remission longer than the first would be an indication of efficacy.

16.21.6.3 Companion Diagnostic for Treatment of Lymphoma with Brentuximab Vedotin

Brentuximab vedotin (Seattle Genetics' Adcetris) is an antibody–drug conjugate (ADC), which comprises an anti-CD30 MAb attached by a protease-cleavable linker to a microtubule-disrupting agent, monomethyl auristatin E (MMAE). The ADC employs a linker system that is designed to be stable in the bloodstream but releases MMAE upon internalization into CD30-expressing tumor cells. Adcetris was granted accelerated approval by the FDA in 2011 for treating two types of lymphoma: (1) relapsed Hodgkin lymphoma after failure of autologous stem cell transplant or after failure of at least two prior multiagent chemotherapy regimens and (2) systemic anaplastic large-cell lymphoma after failure of at least one prior multiagent chemotherapy regimen.

16.21.7 Personalized Management of Hepatocellular Carcinoma

Astrocyte-elevated gene-1 (AEG-1) is overexpressed in >90 % of human hepatocellular carcinoma (HCC) patients and plays a significant role in mediating aggressive progression of HCC, a highly fatal cancer with currently very limited therapeutic options. AEG-1 is known to augment invasion, metastasis, and angiogenesis and now has been shown to directly contribute to another important hallmark of aggressive cancers, that is, resistance to chemotherapeutic drugs, such as 5-FU (Yoo et al. 2009). AEG-1 augments expression of the transcription factor LSF that regulates the expression of thymidylate synthase, a target of 5-FU. In addition, AEG-1 enhances the expression of dihydropyrimidine dehydrogenase (DPD) that catalyzes the initial and rate-limiting step in the catabolism of 5-FU. siRNA-mediated inhibition

of AEG-1, LSF, or DPD significantly increases the sensitivity of HCC cells to 5-FU in vitro and a lentivirus delivering AEG-1 siRNA in combination with 5-FU markedly inhibited growth of HCC cells xenotransplanted in athymic nude mice when compared to either agent alone. Thus AEG-1 and LSF genes contribute to chemoresistance. Inhibition of AEG-1 can be exploited as a therapeutic strategy along with 5-FU-based combinatorial chemotherapy for HCC.

16.21.8 Personalized Management of Gastrointestinal Cancer

16.21.8.1 Personalized Management of Esophageal Cancer

Esophageal cancer is highly aggressive malignancy. Almost half of new cases are diagnosed at an advanced stage, when the 5-year survival rate is just 14 %. Surgery is offered to most patients, as well as one or all of the following treatments: an antimetabolite chemotherapy agent (5-FU), an alkylating agent (cisplatin), and a radiation treatment. Several gene variants can predict an improved outcome in patients treated with two different chemotherapy drugs and/or with radiation therapy. Application of pharmacogenetic analysis to multiple genes in each drug action pathway to patients with resectable adenocarcinoma or squamous cell carcinoma of the esophagus, who had been treated with chemoradiation followed by esophagectomy, shows that methylenetetrahydrofolate reductase polymorphisms can modify 5-FU response. This supports the hypothesis that response or resistance to therapy in esophageal cancer patients may be modulated by genetic variants involved in the metabolism or mechanism of chemotherapy drug action. The ongoing esophageal cancer research aims to determine individual pharmacogenetic profiles to identify patients most likely to have chemotherapeutic benefit and patients with the highest risk of suffering genotoxic side effects. These profiles will ideally lead to individualized therapies, improved treatment outcomes, and a movement toward clinically applied pharmacogenetics. This emergent area of biomedicine could lead to substantially improved clinical outcomes for patients with adenocarcinoma or squamous cell carcinoma of the esophagus. For example, a combination of several gene variants in patients treated with one type of chemotherapy (5-FU) more than doubled survival in patients treated with the same drug who did not have these variants. The findings represent a significant advance in the goal to provide personalized therapy because it offers a genetic blueprint for gauging the potential effectiveness of all common esophageal cancer treatment, not just an analysis of how one or two "candidate" genes respond to a single treatment. The patients with the best outcomes were those who had gene variants that were less effective at neutralizing the killing power of the cancer treatments. Conversely, patients whose genes efficiently counteracted chemotherapy and radiation treatment had shorter survival times overall. Another finding of the study was an additive effect between these genes and others that conferred smaller advantages. The higher the number of beneficial variants the patient had, the longer survival was. If successful, such pathway-based analyses can be conducted for the wide variety of cancers that are treated with 5-FU, cisplatin, and radiation, as well as other drug treatments.

16.21.8.2 Personalized Management of Gastric Cancer

Despite considerable improvements in surgical techniques, innovations in clinical diagnostics, and the development of new chemotherapy regimens, the clinical outcome for patients with advanced gastric cancer is generally poor with 5-year survival rates ranging between 5 and 15 %. Several molecular therapies are in development for gastric cancer. Cyclooxygenase-2 (COX-2) is overexpressed in and correlated with gastric cancer, and knockdown of COX-2 or administration of COX-2 inhibitors suppresses tumor formation in models of gastric cancer. Induction of apoptosis, reduction of angiogenesis, and blocking of potassium ion channels may present new mechanisms of COX-2 inhibition. Osteopontin is a secreted protein involved in stress response, inflammation, wound healing, and immune response. Inhibition of osteopontin by RNAi technique suppresses tumorigenesis as well as angiogenesis in gastric cancer. Using HLA-A-matched allogeneic gastric cancer cells to induce tumor-specific CTLs is another option for personalized immunotherapy of gastric cancer (Wu et al. 2009). In ~22 % of cases, gastric cancer is HER2-positive. A phase III study has shown that Herceptin (Genentech/Roche) is effective in advanced HER2-positive stomach cancer.

16.21.8.3 Personalized Management of Colorectal Cancer

The cause of CRC is multifactorial, involving hereditary susceptibility, environmental factors, and somatic genetic changes during tumor progression. Due to a high degree of genetic and pathological heterogeneity, sporadic CRC is considered a collection of diseases that should be approached with different therapeutic strategies. Integration of a new generation of molecularly targeted drugs into the treatment of CRC, coupled with the development of sophisticated technologies for individual tumors as well as patient molecular profiling, forms the bases of personalized management of CRC.

Hereditary nonpolyposis CRC (HNPCC) is a familial cancer syndrome characterized by mutations in at least one of six DNA mismatch repair genes: hPMS1, hPMS2, hMSH2, MSH6, hTGFBR2, and hMLH1. From 5 to 10 % of the 150,000 cases of CRC diagnosed each year in the USA are of hereditary type. The identification of DNA MSI refines the diagnosis of HNPCC, allowing frequent early-onset colonoscopic screening to be restricted to individuals with an especially high risk of this type of cancer. It is possible that a combination of tests for MSI, allelic loss, p53 mutations, and other genetic alterations in patients with early-stage CRC will define groups of patients who require different adjuvant therapies or no systemic treatment at all. Despite the recent results of systemic chemotherapy, more than 40 % of patients with advanced cancer still do not achieve substantial benefits with cytotoxic agents. Therefore, personalized strategies are warranted to improve the probability of disease control. It is important to have a strategy for screening and early detection for preventive measures.

The NCI has developed absolute risk prediction models for CRC from population-based data and a simple questionnaire suitable for self-administration

(Freedman et al. 2009). The model included a cancer-negative sigmoidoscopy/colonoscopy in the last 10 years, polyp history in the last 10 years, history of CRC in first-degree relatives, aspirin and NSAID use, hormone use, cigarette smoking, body mass index, current leisure-time vigorous activity, and vegetable consumption (www.cancer.gov/colorectalcancerrisk). The absolute risk model for CRC was well calibrated in a large prospective cohort study (Park et al. 2009a). This prediction model, which estimates an individual's risk of CRC given age and risk factors, may be a useful tool for physicians, researchers, and policymakers.

The success of chemotherapy depends on various factors such as gender, age, and histological subtype of tumor. The difference in drug effects between different genotypes can be significant. Promising candidates have been identified with predictive value for response and toxicity to chemotherapy in CRC. These candidates need to be incorporated into large, prospective clinical trials to confirm their impact for response and survival to chemotherapy that has been reported in retrospective analyses. Confirmed predictive markers, together with additional yet to be identified pharmacogenomic key players, will provide the basis for tailoring chemotherapy in the future. The rationale for this approach is based on the identification of the in vivo interactions among patient's characteristics, disease physiopathology, and drug pharmacodynamics and pharmacokinetics. Despite the recent encouraging data, the clinical use of targeted therapy is hampered by several questions that need to be answered such as optimal biologic dose and schedule, lack of predictive surrogate biomarkers, and modalities of combination with chemotherapy/radiotherapy. To improve this situation, high-throughput methods have been used to discover prognostic and predictive biomarkers for CRC. There is still a need for multiple biomarker testing and for identifying panels of predictive biomarkers in order to improve response rates and decrease toxicity with the ultimate aim of tailoring treatment according to an individual patient and tumor profile. Three major genetic and epigenetic alterations that drive CRC tumorigenesis have been identified: MSI, CIN, and CpG island methylator phenotype (CIMP). These alterations have mainly been used as biomarkers for defining CRC prognosis, but recent data have demonstrated their correlation with treatment response. Utilization of K-ras gene status as a therapeutic biomarker for the administration of EGFR inhibitors is a notable example of how molecular profiling can provide unique advantages for the identification of subpopulations of patients with a high response rate to standard of care.

Most of the targeted inhibitors in development or in clinical use are molecules with high affinity for growth factor receptors, such as FGFR, VEGFR, PDGFR, mast/stem cell growth factor receptor (KITR), and EGFR. Introduction of MAbs that bind to growth factors into the combination chemotherapy regimens currently used in metastatic CRC has been shown to be effective and has further widened the treatment options. The present scientific consensus is that the large individual differences in treatment response among CRC patients are due to the fact that each patient's tumor is different at the molecular level as a result of the unique genetic and environmental background of that patient (Silvestri et al. 2013). Therefore, an understanding of these molecular differences is essential for optimizing treatment regimens. For this reason, the development and application of individualized therapy have been the goal of several studies within the last decade.

DNA microarray analysis was used to analyze the transcriptional profile of HCT116 CRC cells that were treated with 5-FU or oxaliplatin and selected for resistance to these agents (Boyer et al. 2006). Bioinformatic analyses identified sets of genes that were constitutively dysregulated in drug-resistant cells and transiently altered following acute exposure of parental cells to drug. Functional analysis of three genes identified in the microarray study (prostate-derived factor, calretinin, and spermidine/spermine N1-acetyltransferase) revealed their importance as novel regulators of cytotoxic drug response. These data show the power of this novel microarray-based approach to identify genes which may be important biomarkers of response to treatment and/or targets for CRC.

Panitumumab (Amgen's Vectibix) is a recombinant, human IgG2 kappa MAb that binds specifically to the human EGFR and is indicated as a single agent for the treatment of EGFR-expressing, metastatic CRC with disease progression on or following fluoropyrimidine-, oxaliplatin-, and irinotecan-containing chemotherapy. A companion diagnostic, TheraScreen K-ras Mutation Kit (DxS Ltd.), which was used in the pivotal clinical trial for panitumumab, is available in 22 EU countries. The kit detects seven mutations in codons 12 and 13 of the K-ras oncogen. Patients with CRC-bearing mutated K-ras do not benefit from cetuximab, whereas patients with a tumor-bearing wild-type K-ras do benefit from cetuximab (Karapetis et al. 2008). The mutation status of the K-ras gene has no influence on survival among patients treated with best supportive care alone. Launch of this companion diagnostic in 2008 marked the first time that the European Commission licensed a bowel cancer treatment with the stipulation that a predictive test should be carried out. In 2009, the FDA updated the "indication and usage" section of the labels of Vectibix and Erbitux (ImClone/Bristol-Myers Squibb) to note that retrospective analyses of metastatic CRC have not shown a treatment benefit for the EGFR inhibitors in patients whose tumors had K-ras mutations in codon 12 or 13 and that the use of the drugs is not recommended for the treatment of CRC patients with these mutations. Physicians can now eliminate anti-EGFR antibodies as a treatment option for patients with mutated K-ras tumors and redirect those patients to alternative therapies, avoiding unnecessary treatments in patients who are unlikely to benefit.

In general, CRC prognosis is based on clinical staging, with roughly 40 % of cases diagnosed in early or localized stages. Patients with stage I and II colon cancer are often considered cured following surgery. Nevertheless, some 15–20 % of these individuals eventually have recurrence of the disease. Therefore, efforts are being made to define the molecular changes associated with recurrence and decreased survival. Since high tumor grade is not a biomarker of higher recurrence risk in stage II colon cancer, it suggests that other biomarkers, such as Genomic Health's Oncotype DX® Recurrence Score® test as well as T-stage and mismatch repair status, should be considered during the treatment decision-making process.

Interest is focused on DNA methylation, an epigenetic mechanism that is involved in everything from imprinting to X-chromosome inactivation, for determining prognosis of CRC. The results of an analysis of the methylation patterns using pyrosequencing in CRC samples taken from two independent prospective cohorts suggest that decreased methylation in regions of the genome called long interspersed nucleotide element-1 (LINE-1) elements is independently associated

with poor survival outcomes (Ogino et al. 2008). A 30 % decrease in LINE-1 methylation doubled the risk of CRC-specific mortality, and the lower the methylation level, the worse the patient outcomes. Methylation changes associated with mortality may reflect genomic instability, transcriptional dysregulation, and the activation of oncogenes, inflammation, or oxidative stress. Although follow-up studies are still needed, there are good prospects of clinical application of the results.

Another study has identified a 50-gene signature in early-stage colon cancer that predicts cancer recurrence (Garman et al. 2008). The investigators compiled gene expression data from publicly available datasets, assessing the expression patterns in 52 samples taken from individuals with known survival outcomes. This signature included RAS and TNF family genes previously implicated in carcinogenesis as well as genes in several pathways linked to metastasis. The team validated nine of the top ten differentially expressed genes using RT-PCR. Along with its prognostic implications, preliminary results suggest that the signature, which was validated in two independent patient groups, may also provide clues for treating colon cancer. Examination of gene expression in early-stage CRC revealed certain patterns that seem to put some patients at higher risk for recurrence. The signature could detect recurrence with more than 90 % accuracy regardless of the early colon cancer's tumor, node, metastasis, or TNM stage. The identification of these patients may enable targeted and proactive treatment to prevent this recurrence. The investigators also tested whether the gene signature was useful for guiding individuals' treatment and identifying new drugs. Using the Broad Institute's Connectivity Map, they assessed the gene expression profiles of cells treated with a range of drugs to look for profiles resembling the cancer recurrence signature. Their research suggests that at least four drugs may influence the genes involved in the recurrence signature. Subsequent experiments indicated that cell lines with the high recurrence risk signature are sensitive to at least two of these compounds: the COX-2 inhibitor celecoxib and the PI3K inhibitor LY-294002. That, in turn, suggests it may be useful to test the treatments in those with the high-risk signature in order to identify patients who may benefit from such treatments rather than standard chemotherapy. This will individualize the treatment plans for patients with CRC and improve survival. Clinical trials are planned to test this.

The identification of genetic factors underlying drug response in CRC still remains a promising area for improving management of CRC patients. Genetic variations identified in genes encoding thymidylate synthase, DPD, glutathione S-transferase pi, and uridine diphosphate glucosyltransferase 1A1 seem to be promising predictors of drug efficacy and/or toxicity in CRC (Fogli and Caraglia 2009). Additional investigation is needed to validate the clinical relevance of individual genetic differences.

In 2011, OncoTrack, an international consortium of academic researchers, pharmaceutical companies, and commercial partners, launched a 5-year project to develop and assess new biomarkers for CRC. Total budget for the project along with funding from the Innovative Medicines Initiative—a private–public partnership between the pharmaceutical industry and the European Union—amounts to €25.8 million ($35.6 million). OncoTrack was founded to create next-generation methods of biomarker development to develop personalized treatment of CRC.

The consortium, led by Bayer HealthCare Pharmaceuticals and the Max Planck Institute for Molecular Genetics in Germany, includes AstraZeneca, Boehringer Ingelheim, Janssen Pharmaceutica, Merck, Pfizer, and Roche Diagnostics. Academic partners include Uppsala University, University College London, Paris South University, Charité Universitätsmedizin Berlin, Medizinische Universität Graz, and Technische Universität Dresden. International Prevention Research Institute, Experimental Pharmacology and Oncology, and Alacris Theranostics also are members of OncoTrack. The consortium's first project called "Methods for systematic next-generation oncology biomarker development" will seek to generate high-quality genomic and epigenetic data from clinically well-defined CRC tumors and their metastases. The data will be compared to the germ line genome of the patients and will be complemented by a detailed molecular characterization of the tumors. OncoTrack will establish and characterize a new series of xenograft tumor models and cell lines derived from the same set of tumors in order to support tumor biology research and the early stages of biomarker qualification. The combined data from all phases of the project will enable OncoTrack to address fundamental questions regarding the relationship between tumor genotype and phenotype, thus providing the starting point for discovery and selection of suitable candidates for development as biomarkers of CRC.

16.21.8.4 Sequencing for Personalized Management of Colorectal Cancer

The Cancer Genome Atlas project plans to profile genomic changes in 20 different cancer types and has now presented results from multidimensional analyses of human CRC. The distinction between the colon and the rectum is largely anatomical, but it has both surgical and radiotherapeutic management implications as well as an impact on prognosis. Most investigators divide CRC biologically into those with MSI (located primarily in the right colon and frequently associated with the CIMP and hypermutation) and those that are microsatellite stable but chromosomally unstable.

Previous investigations have uncovered several critical genes and pathways that are important in the initiation and progression of CRC. These include the WNT, RAS-MAPK, PI3K, TGF-β, P53, and DNA mismatch repair pathways. Large-scale sequencing analyses have identified numerous recurrently mutated genes and a recurrent chromosomal translocation, but a fully integrated view of the genetic and genomic changes and their significance for CRC tumorigenesis was lacking. Genomic patterns that have now been uncovered in CRC, including samples originating at sites in either the colon or the rectum, reveal genomic profiles that are similar to those present in tumors at each site (The Cancer Genome Atlas Network 2012). By doing sequencing, CNV analyses, and/or methylation profiling on almost 300 CRC samples, the team narrowed in on key genes and pathways that tend to be altered in CRC. For the new analysis, researchers used SOLiD or Illumina sequencing platforms to sequence the exomes of 224 tumor–normal pairs to an average depth of >20× over 80 % or more of the coding sequences targeted. With the Illumina HiSeq 2000, they also did low-coverage WGS on 97 of the tumors and matched normal samples. The transcriptional analysis was expanded further through

RNA sequencing and miRNA sequencing experiments. The data presented provide a useful resource for understanding CRC and identifying possibilities for treating it in a targeted way. Although it may take years to translate this foundational genetic data on CRC into new therapeutic strategies and surveillance methods, this genetic information will be the springboard for determining what will be clinically effective against CRC. A subset of the CRC, most often tumors showing up in the right or ascending colon, had unusually high mutation levels. Approximately 16 % of the tumors could be classified as hypermutated, containing a median of 728 predicted somatic mutations apiece.

More than 75 % of these hypermutated samples showed enhanced methylation levels and MSI. As the survival rate of patients with high MSI-related cancers is better and these cancers are hypermutated, mutation rate may be a better prognostic indicator.

From their genome sequence data, researchers tracked down several suspected translocation events involving bits of sequence from different chromosomes. For example, three of the 97 tumors assessed by low-coverage genome sequencing contained a fusion linking the first exons of the chromosome 11 gene NAV2 to part of the chromosome 2 gene TCF7L1, which codes for a component in the WNT pathway, a known contributor to CRC. Almost all of the tumors from both the hypermutated and the non-hypermutated groups included mutations expected to boost the activity of the WNT signaling pathway and to curb signaling via the TGF-β pathway, changes that are expected to produce an increase in the activity of the myc proto-oncogene. These findings fit with early reports suggesting that compounds targeting that pathway may be effective against some CRCs. Possible therapeutic approaches to CRC included WNT-signaling inhibitors and small-molecule β-catenin inhibitors, which are showing initial promise. Other commonly affected pathways included the RTK-RAS, MAP kinase, TP53, and PI3-kinase pathways, which point to potential targets for new CRC treatments. Approximately 5 % of the CRC tumors studied had extra copies of a gene, ERBB2, as do many breast cancer tumors. The drug, Herceptin, which is effective for breast cancer patients with too many ERBB2 genes, might also help CRC patients with the same aberration. Clinical trials have been proposed to test the effects of Herceptin in these CRC patients. Approximately 15 % of CRCs had a mutation in a gene, BRAF, that is also often mutated in melanoma. A drug approved for melanoma blocks the function of BRAF gene product, but it has not worked in CRC patients.

16.21.8.5 Systems Biology Approach to Drug Resistance in Colorectal Cancer

Mechanisms that may have important implications for drug efficacy and actively contribute to innate resistance in CRC are:

- High levels of thymidylate synthase, the 5-FU target, are associated with tumor insensitivity to FU-based therapy.
- Higher levels of TOP1 correlate with greater sensitivity of colon tumors to camptothecin derivatives compared to normal colonic mucosa.

- Glucuronidation, involved in xenobiotic detoxification, is also associated with innate resistance to TOP1 inhibitors in colon cell lines and tumors.
- An increase of the ABCB1/P-gp transporter, a member of the family of ABC transporters that detect and eject anticancer drugs from cells, is observed in intrinsically drug-resistant colon tumors.

In a systems biology approach to understand innate CRC tumor responses to an FOLFIRI-combined chemotherapy of irinotecan (CPT-11) plus 5-FU/FA, gene expression patterns obtained with microarrays were compared between clinical samples from colon tumors and liver metastases collected from CRC patients prior to drug exposure (Grauden et al. 2006). The use of a vigilant experimental design, power simulations, and robust statistical analysis reduced the false-negative and false-positive differential hybridization rates to a minimum. Data collected from a biological systems perspective into global and interconnected molecular networks highlight the molecular mechanisms that may anticipate resistance in CRC patients prior to their exposure to drugs. This knowledge could be used in clinical practice as a complement to clinical, biochemical, and genetic biomarkers for global prevention, early diagnosis, and better patient treatment.

16.21.8.6 Resistance to Targeted EGFR Blockade in CRC

CRC that is wild type for K-ras is often sensitive to EGFR blockade but almost always develops resistance within several months of initiating therapy. This situation is in marked contrast to that of small-molecule targeted agents, such as inhibitors of ABL, EGFR, BRAF, and MEK, in which mutations in the genes encoding the protein targets render the tumors resistant to the effects of the drugs. Two studies have provided an explanation of why solid tumors develop resistance to targeted therapies in a highly reproducible fashion and provide a basis for overcoming this. One study of circulating tumor DNA found that 38 % of patients whose tumors were initially K-ras wild type developed detectable mutations in K-ras in their sera and some of them developed multiple different K-ras mutations (Diaz et al. 2012). The appearance of these mutations was very consistent, generally occurring between 5 and 6 months following treatment. Mathematical modeling indicated that the mutations were present in expanded subclones before the initiation of panitumumab treatment. These results suggest that the emergence of K-ras mutations is a mediator of acquired resistance to EGFR blockade and that these mutations can be detected in a noninvasive manner.

A second study found that cetuximab, an MAb that binds the ECD of EGFR, is effective in a subset of K-ras wild-type metastatic CRC, but after an initial response, secondary resistance invariably ensues, thereby limiting the clinical benefit of this drug. Further investigations showed that point mutations of K-ras are causally associated with the onset of acquired resistance to anti-EGFR treatment in CRC, but resistant cells remained sensitive to combined inhibition of EGFR and mitogen-activated protein kinase kinase (Misale et al. 2012). Analysis of metastases from patients who developed resistance to cetuximab or panitumumab showed the

emergence of K-ras amplification in one sample and acquisition of secondary K-ras mutations in 60 % of the cases. K-ras mutant alleles were detectable in the blood of cetuximab-treated patients as early as 10 months before radiographic documentation of disease progression. In summary, the results identify K-ras mutations as frequent drivers of acquired resistance to cetuximab in CRC, indicate that the emergence of K-ras mutant clones can be detected noninvasively months before radiographic progression, and suggest early initiation of an MEK inhibitor as a rational strategy for delaying or reversing drug resistance.

16.21.9 Personalized Management of Liver Cancer

Potentially curative treatments of HCC include surgical resection and transplantation. IFN has a significant beneficial effect after curative treatment of HCC in terms of both survival and tumor recurrence. A multikinase inhibitor sorafenib has also been reported to enhance survival. Despite several treatment options, fewer than half of candidates for potentially curative treatments receive them.

Targeted adjuvant therapies require methods to guide the selection of optimal treatment for HCC. HCC can be classified in molecular classes according to Wnt-beta-catenin pathway activation, proliferation signature activation (associated with CIN), and other subgroups. A molecular classification is essential to enable the development of new targets and to customize therapies in patients with HCC (Villanueva et al. 2008).

The expression patterns of miRNAs in liver tissue differ between men and women with HCC. The miR-26 expression status of such patients is associated with survival and response to adjuvant therapy with IFN-α. A study showed that patients whose tumors had low miR-26 expression had shorter overall survival but a better response to IFN-α than patients whose tumors had high expression of the miRNA (Ji et al. 2009). This has implications for the personalized management of liver cancer.

16.21.10 Personalized Management of Lung Cancer

Treatment of lung cancer is considered according to the type; ~85 % have NSCLC, who often present with advanced disease and a low survival rate. It is important to have targeted therapies as well as companion diagnostics to guide the selection of patients most likely to respond to treatment.

16.21.10.1 Crizotinib for Personalized Management of NSCLC

Crizotinib (Pfizer's Xalkori) is an orally available small molecule that blocks fusion proteins of ALK. This fusion, EML4 (echinoderm microtubule-associated protein-like 4)–ALK, is a tyrosine kinase-like BCR–ABL, and its formation drives tumor

formation; therefore, blocking it should halt tumor growth. In a study of 1,500 patients with NSCLC, 5.5 % had a fusion containing ALK, and 57 % of them responded well to crizotinib (Kwak et al. 2010). The FDA has cleared for marketing Abbott's companion diagnostic kit, Vysis ALK Break Apart FISH Probe, for use with crizotinib to detect rearrangements of the ALK gene in NSCLC. The kit uses FISH technology to detect rearrangements on the ALK gene on the 2p23 chromosome, providing clinicians a standardized, clinically validated method to identify those patients who may benefit the most from Pfizer's drug. The test is designed to identify the 3–5 % of all NSCLC patients who would be candidates for Xalkori, and this is expected to change how patients with NSCLC are diagnosed and treated.

Despite these remarkable initial responses, cancers eventually develop resistance to crizotinib, usually within 1 year, thereby limiting the potential clinical benefit. A study enumerates the mutations in EML4–ALK that confer resistance to crizotinib (Choi et al. 2010). Using a model of acquired resistance to ALK inhibitors, it was shown that second-generation ALK TKIs or Hsp90 inhibitors are effective in treating crizotinib-resistant tumors harboring secondary gatekeeper mutations (Katayama et al. 2011).

16.21.10.2 Determination of Outcome of EGFR Tyrosine Kinase Inhibitor Treatment

The TKI gefitinib (Iressa), which targets the EGFR, is approved for late cases of NSCLC as a last resort treatment. Most of NSCLC patients do not respond to gefitinib, but about 10 % of patients have a rapid and often dramatic clinical response. The molecular mechanisms underlying sensitivity to gefitinib are unknown. It was considered to be a targeted therapy based on the idea that lung cancer might make excess EGFR, and blocking it might slow growth with less toxicity than standard chemotherapy. This growth protein contains a little pocket to capture ATP. Gefitinib apparently targets that pocket, and when the protein is mutated, gefitinib fits inside the pocket much better, blocking ATP and thus inhibiting cancer cell growth. Response of lung cancer patients to gefitinib is determined by a certain mutation in the EGFR gene. Patients with lung cancer who respond to gefitinib have been reported to have somatic mutations consisting of deletions in exon 19 and in exon 21 of the EGFR gene. In addition, a mutation in exon 20 is also associated with acquired resistance to gefitinib in initially gefitinib-sensitive patients.

Laboratory studies of cancer cells show that the mutated receptors are 10 times more sensitive to gefitinib than were normal receptors. The mutations are more common in women, people who had never or not recently smoked, and people who have a subtype called bronchoalveolar cancer. EGFR mutations are found in lung cancer samples from patients who responded to gefitinib (Eli Lilly & Co's Iressa) therapy and in a LAD cell line that are hypersensitive to growth inhibition by gefitinib, but not in gefitinib-insensitive tumors or cell lines. These results suggest that EGFR mutations may predict sensitivity to gefitinib. Increased EGFR gene copy number based on FISH analysis is a good predictive biomarker for response to EGFR inhibitors, stable disease, time to progression, and survival in NSCLC.

However, EGFR mutation is a better predictor of clinical outcome in gefitinib-treated patients than the EGFR gene copy number. These findings are important as they would enable the development of personalized treatment of cancer. The EGFR Mutation Assay (Genzyme) detects EGFR mutations in patients with NSCLC that correlate with clinical response to Tarceva® (erlotinib) and Iressa® (gefitinib). This would enable treatment of responders and even at an earlier stage than the current practice of using it as a last resort. Prospective large-scale clinical studies must identify the most optimal paradigm for the selection of patients.

Another drug targeting the EGFR receptor is erlotinib (Tarceva; OSI Pharmaceuticals/Pfizer). A randomized, placebo-controlled, double-blind trial was conducted to determine whether erlotinib prolongs survival in NSCLC after the failure of first-line or second-line chemotherapy (Shepherd et al. 2005). The presence or absence of EGFR mutation was not taken into consideration. The results show that erlotinib can prolong survival in patients with NSCLC after first-line or second-line chemotherapy. A clinical trial has compared responsiveness to erlotinib with a placebo for NSCLC using tumor-biopsy samples from participants in this trial to evaluate EGFR expression immunohistochemically (Tsao et al. 2005). The results indicate that among patients with NSCLC who receive erlotinib, the presence of an EGFR mutation may increase responsiveness to the agent, but it is not indicative of a survival benefit.

Many patients with NSCLC who show radiographic responses to treatment with EGFR TKIs gefitinib and erlotinib have somatic mutations in the EGFR tyrosine kinase domain. Both are known as small-molecule drugs that can be taken orally and block the part of the EGFR molecule that's located within the cell. Studies with gefitinib and cetuximab (Erbitux), an MAb for colon cancer, have shown that although both drugs kill cells containing a normal but overactive EGFR molecule, only gefitinib kills lung cancer cells containing a mutated EGFR molecule, whereas cetuximab has little effect on the mutant signal, evidently because it strikes at a different part of the EGFR molecule. Thus those with EGFR mutations will benefit from gefitinib or erlotinib, while another group, without EGFR mutations, will benefit from cetuximab. Cetuximab binds to a portion of the EGFR receptor that extends outside the cell. This difference in action is the apparent explanation for why they performed differently against the mutant EGFR cells. These studies show that in order to inhibit the mutant receptor, one should inhibit the domain of the EGFR molecule that lies within the cell, as opposed to the ECD.

Previously, tumor biopsies have been used in NSCLC for EGFR genotyping as it has been difficult to detect the low levels of specific mutations shed from the tumor into the blood against the high background of normal DNA. Testing DNA isolated from blood, rather than tumor tissue, would be better for predicting responses to gefitinib, erlotinib (Tarceva), and other cancer therapies. If EGFR mutations can be observed in serum DNA, this could serve as a noninvasive source of information on the genotype of the original tumor cells as compared to direct sampling of the tumor and could influence treatment and the ability to predict patient response to gefitinib. In one study, serum genomic DNA was obtained from Japanese patients with NSCLC before first-line gefitinib monotherapy (Kimura et al. 2006). Scorpion

Amplified Refractory Mutation System technology (DxS Ltd.) was used to detect EGFR mutations. In pairs of tumor and serum samples obtained from patients, the EGFR mutation status in the tumors was consistent with those in the serum of over 72 % of the paired samples. The DxS test kit detected mutations that were missed by direct sequencing techniques. These results suggest that patients with EGFR mutations seem to have better outcomes with gefitinib treatment, in terms of progression-free survival, overall survival, and response, than those patients without EGFR mutations.

TheraScreen EGFR 29 Mutation Test (DxS) detects mutations that correlate with responsiveness to EGFR TKIs. This test may be used to help physicians choose lung cancer patients who are most likely to respond to treatment with EGFR TKIs.

In another approach to this problem, serum collected from NSCLC patients before treatment with gefitinib or erlotinib was analyzed by MALDI MS, and spectra were acquired independently at two institutions (Taguchi et al. 2007). An algorithm to predict outcomes after treatment with EGFR TKIs was developed from a training set of patients from three cohorts. The algorithm was then tested in two independent validation cohorts of patients who were treated with gefitinib and erlotinib and in three control cohorts of patients who were not treated with EGFR TKIs. The clinical outcomes of survival and time to progression were analyzed. This MALDI MS algorithm was not merely prognostic but could classify NSCLC patients for good or poor outcomes after treatment with EGFR TKIs. This algorithm may thus assist in the pretreatment selection of appropriate subgroups of NSCLC patients for the treatment with EGFR TKIs. The test is commercially in development by Biodesix Inc.

One study involving EGFR mutational analysis on DNA recovered by CTC-Chip from CTCs using allele-specific PCR amplification has compared the results with those from concurrently isolated free plasma DNA and from the original tumor-biopsy specimens (Maheswaran et al. 2008). Thus molecular analysis of CTCs from the blood of patients with lung cancer offers the possibility of monitoring changes in epithelial tumor genotypes during the course of treatment.

A study from Spain has evaluated the feasibility of large-scale screening for EGFR mutations in advanced NSCLC and analyzed the association between the mutations and the outcome of erlotinib treatment (Rosell et al. 2009). It concluded that large-scale screening for EGFR mutations is feasible with subsequent customization of erlotinib and improved outcome. It is warranted in women with lung cancer, in those who have never smoked, and in those with nonsquamous tumors.

16.21.10.3 Development of Resistance to EGFR Inhibitors

Acquired resistance to EGFR TKIs is inevitable in metastatic EGFR-mutant lung cancers. Because RAS/RAF/MEK mutations are known mediators of acquired resistance in other solid tumors (colon cancers, GISTs, and melanomas) responsive to targeted therapies, the frequency of secondary K-ras/NRAS/BRAF/MEK1 gene mutations was analyzed in the largest collection to date of lung cancers with acquired

resistance to EGFR TKIs (Ohashi et al. 2012). No recurrent NRAS, K-ras, or MEK1 mutations were found in most of patient samples, but 1 % were found to have mutations in BRAF. Ectopic expression of mutant NRAS or BRAF in drug-sensitive EGFR-mutant cells conferred resistance to EGFR TKIs that was overcome by addition of an MEK inhibitor. Collectively, these positive and negative results provide deeper insight into mechanisms of acquired resistance to EGFR TKIs in lung cancer and should be taken into consideration in ongoing clinical trials designed to overcome resistance. In the context of emerging knowledge about mechanisms of acquired resistance to targeted therapies in various cancers, these data highlight that, even though solid tumors share common signaling cascades, mediators of acquired resistance must be elucidated for each disease separately in the context of treatment.

16.21.10.4 Molecular Subtyping of Lung Cancer

LAD has extreme genetic variation among patients, which is not well understood and limits progress in research and development of therapy. LAD molecular subtypes are a validated stratification of naturally occurring gene expression patterns and encompass different functional pathways and patient outcomes. Different subtypes may be the result of mutations and alterations in gene expression. LAD molecular subtypes (Bronchioid, Magnoid, and Squamoid) were tested for association with gene mutations and CNVs using statistical methods and published cohorts (Wilkerson et al. 2012). A novel validation cohort was assayed and interrogated to confirm subtype–alteration associations. Mutation rates of genes (EGFR, K-ras, STK11, TP53), CIN, regional copy number, and genome-wide DNA methylation were significantly different among tumors of the molecular subtypes. Secondary analyses compared subtypes by integrated alterations and patient outcomes. Tumors having integrated alterations in the same gene associated with the subtypes, e.g., mutation, deletion, and underexpression of STK11 with Magnoid and mutation, amplification, and overexpression of EGFR with Bronchioid. The subtypes also associated with tumors having concurrent mutant genes, such as K-ras–STK11 with Magnoid. Overall survival of patients, cisplatin plus vinorelbine therapy response, and predicted gefitinib sensitivity were significantly different among the subtypes. The study concluded that LAD intrinsic molecular subtypes co-occur with grossly distinct genomic alterations that affect response to therapy. These results advance the understanding of etiology of LAD and help in the selection of patient subgroups for future evaluation of treatment response. Lung Subtype Platform (LSP™) is being developed commercially by GeneCentric.

16.21.10.5 Personalized Therapy of NSCLC Based on KIF5B/RET Fusion Oncogene

Although several studies have reported genomic driver mutations in NSCLC over the past decade, the molecular pathogenesis of more than 40 % of NSCLC is still unknown. To identify new molecular targets in NSCLC, the combined analysis of

massively parallel whole-genome and transcriptome sequencing for cancer was performed on an adenocarcinoma patient, who is a nonsmoker and has no family history of cancer (Ju et al. 2012). The cancer showed no known driver mutation in EGFR or K-ras and no EML4–ALK fusion. However, a novel fusion gene was found between KIF5B and RET proto-oncogene, which is caused by a pericentric inversion of 10p11.22-q11.21. This fusion gene overexpresses chimeric RET RTK, which could spontaneously induce cellular transformation. KIF5B–RET fusion has been identified in a few more cases indicating that a subset of NSCLC could be caused by a fusion of KIF5B and RET and suggesting the chimeric oncogene as a promising molecular target for the personalized diagnosis and treatment of lung cancer.

16.21.10.6 Role of a New Classification System in the Management of Lung Cancer

Apart from genotyping, a new staging system that was developed by the International Association for the Study of Lung Cancer will have a considerable impact on the future management of lung cancer. Changes in the new classification include creating more substages for tumor size, reassigning some large tumors to a more advanced stage, reclassifying tumors that have spread into the fluid surrounding the lung, and recognizing that spread to certain lymph nodes is more dangerous than its spread to others. By changing these groupings, some patients will get moved to an earlier stage of disease that may be treated more aggressively. For example, a patient may have only been offered chemotherapy but may now be offered chemotherapy and radiation or more intense radiation. Conversely, some people considered to have earlier-stage tumors now will be grouped with those whose tumors have widely spread and discouraged from undergoing therapies that have little chance of helping them.

16.21.10.7 Selecting Therapy of Cancer Arising from Respiratory Papillomatosis

In a case of recurrent respiratory papillomatosis with progressive, bilateral tumor invasion of the lung parenchyma, conditional reprogramming was used to generate cell cultures from the patient's normal and tumorous lung tissue. Analysis revealed that the laryngeal tumor cells contained a wild-type 7.9-kb HPV type 11 (HPV-11) genome, whereas the pulmonary tumor cells contained a 10.4-kb genome (Yuan et al. 2012). The increased size of the latter viral genome was due to duplication of the promoter and oncogene regions. The spread of the tumor in the lung was most likely due to the distal aspiration of tumor cells rather than reinfection of new cells. Finally, the finding that the laryngeal tumor lacked the 10.4-kb genome suggests that duplication in the viral genome did not precede extension into the lung. Chemosensitivity testing identified vorinostat as a potential therapeutic agent, which led to stabilization of tumor size with durable effects. This is a good example of use of biotechnology to understand the spread of tumor in an individual patient and selection of appropriate therapy.

16.21.10.8 Testing for Response to Chemotherapy in Lung Cancer

To gain insight into clinical response to PBC in NSCLC, matched tumor and non-tumor lung tissues from PBC-treated NSCLC patients—nonresponders as well as responders—and tumor tissue from an independent test set have been profiled using microarrays. Lysosomal protease inhibitors SerpinB3 and cystatin C are highly correlated with clinical response. This pathway within tumor cells is involved in resistance to chemotherapy and prevents PBC from killing cancer cells. Tests based on this mechanism may allow clinicians to predict whether or not a lung cancer patient will respond to chemotherapy and help in decision-making about how the patient could best be treated, therefore, moving lung cancer patients closer to personalized treatments. This finding could also lead to the development of new drugs to target this pathway, which could subsequently lead to more effective treatments for lung cancer.

Polymorphisms in the MDR1 gene may have an impact on the expression and function of P-glycoprotein encoded by it, thereby influencing the response to chemotherapy. Patients harboring the 2677G–3435C haplotype have a statistically significant better response to chemotherapy compared with those with the other haplotypes combined (Sohn et al. 2006). These findings suggest that the MDR1 polymorphisms can be used for predicting treatment response to etoposide–cisplatin chemotherapy in SCLC patients.

16.21.10.9 Testing for Prognosis of Lung Cancer

A substantial number of studies have reported the development of gene expression-based prognostic signatures for lung cancer. The ultimate aim of such studies should be the development of well-validated clinically useful prognostic signatures that improve therapeutic decision-making beyond current practice standards. A review of published articles on gene expression-based prognostic signatures in lung cancer revealed little evidence that any of the signatures are ready for clinical use (Subramanian and Simon 2010). Those publications were evaluated in detail for appropriateness of the study design, statistical validation of the prognostic signature on independent datasets, presentation of results in an unbiased manner, and demonstration of medical utility for the new signature beyond that obtained using existing treatment guidelines. Serious problems in the design and analysis of the studies were also found. The authors suggest a set of guidelines to aid the design, analysis, and evaluation of prognostic signature studies. These guidelines emphasize the importance of focused study planning to address specific medically important questions and the use of unbiased analysis methods to evaluate whether the resulting signatures provide evidence of medical utility beyond standard of care-based prognostic factors.

Pervenio™ Lung RS (Life Technologies), a 14-gene expression assay that uses real-time qPCR and runs on FFPE tissue samples to differentiate patients with heterogeneous statistical prognoses, has been developed for patients with

nonsquamous NSCLC (Kratz et al. 2012). Among patients whom the assay identified as low risk for recurrence, 71.4 % were still alive after 5 years, and among patients it determined to be at intermediate risk for recurrence, 58.3 % survived beyond 5 years. Among high-risk patients, 49.2 % survived beyond 5 years. It reliably identifies patients with early-stage nonsquamous NSCLC at high risk for mortality after surgical resection, and it provides prognostic differentiation of patients with early-stage disease, which might be helpful in the identification of the most appropriate application of treatment guidelines to improve clinical outcomes.

Sixteen genes that correlate with survival among patients with NSCLC were identified by analyzing microarray data and risk scores. A test based on five of these (DUSP6, MMD, STAT1, ERBB3, and LCK) using RT-PCR and decision-tree analysis was shown to correlate with relapse-free and overall survival among patients with NSCLC (Chen et al. 2007). This five-gene signature test can be used for predicting survival.

16.21.11 Personalized Management of Malignant Melanoma

If detected early, melanoma carries an excellent prognosis after appropriate surgical resection, whereas advanced melanoma has a poor prognosis and is notoriously resistant to radiation and chemotherapy. There are few effective therapies for metastatic melanoma. Two therapies approved by the FDA, high-dose IL-2 and dacarbazine, are both associated with response rates of 10–20 % and a small percentage of complete responses; neither improves overall survival. The relative resistance of melanoma to a wide range of chemotherapeutic agents and high toxicity of current therapies has prompted a search for effective alternative treatments that would improve prognosis and limit side effects.

Personalized medicine has long been a mainstay of the treatment of localized melanoma, involving surgical decisions that are individualized on the basis of measured differences as small as 0.01 mm, as well as other biomarkers of metastatic potential, such as the presence of ulceration or mitoses. The genetic characterization of primary tumors as well as hereditary susceptibility to melanoma opens the door for tailored pharmacologic therapy. Genetic testing for CDKN2A and CDK4 is already available. Genetic tests for ARF and MC1R are likely to be available soon to evaluate an individual's hereditary risk for developing melanoma.

Several pharmacogenomic-based therapies are in development for melanoma. However, once melanoma spreads beyond the regional nodes, the lack of validated molecular targets hampers efforts to individualize therapy. In the past decade, targeted inhibitors have been developed for metastatic melanoma to enable more personalized therapies of genetically characterized tumors. The identification of somatic mutations in the gene encoding the serine/threonine protein kinase BRAF in the majority of melanomas offers an opportunity to test oncogene-targeted therapy for this disease.

16.21.11.1 Inhibitors of BRAF Mutation for Metastatic Melanoma

Mucosal and acral-lentiginous melanomas, comprising 3 % of all melanomas, frequently harbor activating mutations of c-kit and drugs targeting this mutation seem to confer similar benefits for these types of tumors (Puzanov and Flaherty 2010).

V600E mutation of the BRAF serine/threonine kinase is present in more than 50 % of all melanomas. The mutation appeared to confer a dependency by the melanoma cancer cell on activated signaling through mitogen-activated protein kinase pathway. The frequency and focality of this mutation (>95 % of all BRAF mutations being at V600 position) suggested its importance in melanoma pathophysiology and potential as a target for therapy. Vemurafenib is an orally available inhibitor of mutated BRAF. A phase II clinical trial showed that treatment of metastatic melanoma with vemurafenib in patients with tumors that carry the V600E BRAF mutation resulted in complete or partial tumor regression in the majority of patients (Flaherty et al. 2010). A phase III randomized clinical trial vemurafenib in patients with previously untreated, metastatic melanoma with the BRAF V600E mutation showed improved survival as compared to those treated with dacarbazine (Chapman et al. 2011). A treatment strategy that combines vemurafenib with Yervoy (Bristol-Myers Squibb), an approved melanoma treatment, will further improve outcomes for melanoma patients with BRAF mutation. In a phase III open-label randomized trial, trametinib, as compared with chemotherapy, improved rates of progression-free and overall survival among patients who had metastatic melanoma with a BRAF V600E or V600K mutation (Flaherty et al. 2012).

BRAF inhibitor sorafenib has not shown selective affinity against tumors carrying BRAF mutations in clinical trials, whether used alone or in combination with other chemotherapies. It is possible that non-BRAF side effects of sorafenib limit the likelihood of achieving drug concentrations that are high enough to inhibit V600 mutation.

16.21.11.2 Management of Drug-Resistant Metastatic Melanoma

BRAF inhibitors vemurafenib and dabrafenib markedly inhibit tumor growth and advance patients' overall survival, but this response is almost inevitably followed by complete tumor relapse due to drug resistance hampering the encouraging initial responses. Several mechanisms of resistance within and outside the MAPK pathway have now been uncovered and have paved the way for clinical trials of combination therapies to try and overcome tumor relapse. It is apparent that personalized treatment management will be required in this new era of targeted treatment. CTCs provide an easily accessible means of monitoring patient relapse and several new approaches are available for the molecular characterization of CTCs. Thus CTCs provide a monitoring tool to evaluate treatment efficacy and early detection of drug resistance in real time. Advances in the molecular analysis of CTCs may provide insight into new avenues of approaching therapeutic options that would benefit personalized melanoma management (Klinac et al. 2013).

16.21.11.3 Vaccine for Malignant Melanoma Based on Heat Shock Protein

Autologous tumor-derived HSP gp96 peptide complex (HSPPC-96, Prophage®, vitespen) vaccine (Agenus Inc.) is emerging as a tumor- and patient-specific cancer vaccine, with confirmed activity in several malignancies. It has been tested in phase III clinical trials in advanced melanoma with evidence for efficacy in patients with earlier-stage disease. HSPPC-96-based vaccine demonstrated an excellent safety profile, thus emerging as a novel therapeutic approach with a suggestive role in cancer therapy. Further investigations are needed to understand the biological basis of immune functions in order to improve the clinical outcome of HSP-based cancer therapy. In the near future, the combination of HSP-based vaccines with other biological compounds might represent a successful strategy in the therapy of advanced melanoma.

16.21.12 Personalized Management of Pancreatic Cancer

There are two types of pancreatic cancer: exocrine tumors and neuroendocrine tumors. Exocrine tumors are the majority of pancreatic cancers, and the most common form is an adenocarcinoma, which begin in gland cells, usually in the ducts of the pancreas. These tumors tend to be more aggressive than neuroendocrine tumors, but if detected early enough they can be treated effectively with surgery. Neuroendocrine tumors constitute only 1 % of all pancreatic cancers. They can be benign or malignant, but the distinction is often unclear and sometimes apparent only when the cancer has spread beyond the pancreas. The 5-year survival rate for neuroendocrine tumors can range from 50 to 80 %, compared with less than 5 % for adenocarcinoma. Pancreatic cancer is so lethal because during the early stages, when it would be most treatable, there are usually no symptoms. It tends to be discovered at advanced stages when abdominal pain or jaundice may result. More advanced tumors have a higher risk of recurrence and can spread to the liver. Pancreatic cancer is usually controllable only through removal by surgery and only if found before it has spread. Palliative care can help a patient's quality of life if the disease has spread. The survival rate of pancreatic cancer patients is the lowest among those with common solid tumors, and early detection is one of the most feasible means of improving outcomes. Currently there are no general screening tools.

Two drugs are approved for the treatment of pancreatic neuroendocrine tumors: everolimus and sunitinib malate. Both drugs suppress angiogenesis and metabolism of the tumor cells. This is a progress compared to previous standard of care, which was chemotherapy, but both these drugs can have severe adverse effects. A number of new agents are being looked at in clinical trials that focus on pathways involved in pancreatic cancer. One is an antibody in development by NCI that blocks the protein PD-1 on the surface of pancreatic cancer and would be more effective because it would produce an enhanced immune response against the tumor. Targeted

nanoparticles coated with material that hone in on tumor cells and deliver drugs to kill them are being tested in animal models as treatment for metastatic neuroendocrine tumors. The main advantage would be reducing the toxicity of the drugs to the normal tissues of the body. The future treatment of pancreatic cancer will involve a personalized approach, i.e., matching a patient's particular type of tumor with treatment using genomic information.

16.21.12.1 Histone Modifications Predict Treatment Response in Pancreatic Cancer

An assay to detect histone modifications can now be used to predict prognosis and response to treatment in subsets of patients with pancreatic cancer (Manuyakorn et al. 2010). The scientists used tissues from a cohort of patients enrolled in the radiation therapy oncology group (RTOG) 9704 trial, a multicenter, phase III study of pancreatic cancer comparing adjuvant gemcitabine with 5-FU, and a separate cohort of patients with stage 1 or 2 pancreatic cancer. IHC was performed for histone H3 lysine 4 dimethylation (H3K4me2), histone H3 lysine 9 dimethylation (H3K9me2), and histone H3 lysine 18 acetylation (H3K18ac). Positive tumor cell staining for each histone modification was used to classify patients into low- and high-staining groups, which were related to clinicopathologic parameters and clinical outcome measures. Low cellular levels of H3K4me2, H3K9me2, or H3K18ac were each significant and independent predictors of poor survival. Combined low levels of H3K4me2 and/or H3K18ac were the most significant predictor of overall survival. In subgroup analyses, histone levels were predictive of survival specifically for those patients with node-negative cancer or for those patients receiving adjuvant 5-FU but not gemcitabine in RTOG 9704. The investigators concluded that cellular levels of histone modifications define previously unrecognized subsets of patients with pancreatic adenocarcinoma with distinct epigenetic phenotypes and clinical outcomes and represent prognostic and predictive biomarkers that could form basis of clinical decisions, including the use of 5-FU chemotherapy. Further research in cell lines and animal models will determine what, if any, role the histone modifications have in causing the development of aggressive forms of pancreatic cancer. Uncovering the mechanism of how the histone modifications are associated with cancer development and/or progression may facilitate design of strategies to interfere with that process and form the basis for a targeted therapy or chemoprevention.

16.21.13 Personalized Management of Prostate Cancer

There are several controversies about management of prostate cancer. Molecular diagnostics has been used to guide therapy of prostate cancer.

16.21.13.1 Diagnostics for Guiding Therapy of Prostate Cancer

Testing tissues from men with prostate cancer has demonstrated how the loss of PTEN, a gene that inhibits tumor growth, results in the uncontrolled activation of a tumor-promoting protein, AKT. AKT then activates the enzyme mTOR, which subsequently activates S6. This is the basis of a tumor-promoting cascade, similar to a domino effect. These biomarkers can be used to predict response to an experimental therapy known as CCI-779, an inhibitor of mTOR. A drug that inhibits mTOR should impact the tumor cells but have no effect on the normal cells. When mTOR is inhibited, the cascade comes to a standstill and tumors stop growing. Prior to identifying this method, there was no molecular method to predict which men with prostate cancers would be sensitive to CCI-779. Now oncologists can customize "targeted" cancer treatments for each patient based on the molecular makeup of their tumors. These "smart drugs" selectively stop the growth of tumor cells with the molecular abnormality. Of those, 25–30 % were predicted to have tumors that are missing PTEN. Therefore, the experimental drug could potentially help about 60,000 prostate cancer patients a year, if the laboratory results are confirmed in clinical trials, which are ongoing.

Prostate Px (Aureon Laboratories) integrates histology, molecular biology, and clinical information and applies bioinformatics to stratify patients as high or low risk for disease recurrence postprostatectomy. Results are provided as the Prostate Px score (0–100), which reports the likelihood of recurrence of the prostate cancer. In a prospective study, integration of clinicopathologic variables with imaging and biomarker data (systems pathology) resulted in a highly accurate tool for predicting clinical failure within 5 years after prostatectomy (Donovan et al. 2008). The data support a role for androgen receptor signaling in clinical progression and duration of response to androgen deprivation therapy.

The anticancer agents docetaxel and thalidomide show significant interindividual variation in their pharmacokinetic and toxicity profiles as well as wide pharmacological variations. In one study, patients with prostate cancer enrolled in a randomized phase II trial using docetaxel and thalidomide versus docetaxel alone were genotyped using the Affymetrix DMET 1.0 platform, which tests for 1,256 genetic variations in 170 drug disposition genes (Deeken et al. 2010). Genetic polymorphisms were analyzed for associations with clinical response and toxicity. In all, ten SNPs in three genes were potentially associated with response to therapy: peroxisome proliferator-activated receptor-δ (PPAR-δ), sulfotransferase family, cytosolic, 1C, member 2 (SULT1C2), and carbohydrate (chondroitin 6) sulfotransferase 3 (CHST3). Genotyping results between drug-metabolizing enzymes and transporters (DMET) and direct sequencing showed >96 % of concordance. These findings highlight the role that non-CYP450-metabolizing enzymes and transporters may have in the pharmacology of docetaxel and thalidomide. DMET appears to offer great promise in this field as a reliable test unveiling genetic variations that correlated with drug effectiveness and toxicity.

16.21.13.2 Prolaris Assay for Determining Prognosis in Prostate Cancer

Prolaris test (Myriad), a 46-gene expression assay launched in 2010, is used to predict men who are at a heightened risk of biochemical recurrence of prostate cancer and therefore should receive more aggressive therapy. In 2012, Myriad, in collaboration with Intermountain Healthcare (Salt Lake City, UT), started PRO 008, a project to analyze biopsy samples from 200 patients who have been diagnosed with prostate cancer. The goal is to demonstrate the prognostic ability of Prolaris to assess a patient's risk of biochemical recurrence of disease and death resulting from it.

16.21.13.3 Detection of Prostate Cancer Metastases

Prostate circulating tumor cells (PCTCs) in circulation are shed from either a primary tumor or metastases, which are directly responsible for most prostate cancer deaths. Quantifying exfoliated PCTCs may serve as an indicator for the clinical management of prostate cancer, isolating and removing of PCTCs could potentially reduce prostate cancer metastasis, and culturing and characterizing captured PCTCs could facilitate the development of personalized treatment options. PSMA, an established biomarker for prostate cancer, is strongly expressed on prostate tumor cells associated with high-grade primary, androgen-independent, and metastatic tumors.

Chemoaffinity capture with magnetic beads of pretargeted PCTCs from peripheral blood can serve as an effective tool for the detection of metastatic prostate cancer, the monitoring of treatment, and the development of personalized therapy based on the responsiveness of PCTCs to chemotherapeutic strategies (Wu et al. 2012). MenaCalc™ Prostate (MetaStat Inc.) is a diagnostic for prostate cancer to help in informed decision about whether to undergo radical surgery and risk its dreaded side effects.

16.21.13.4 Early Detection of Cancer Recurrence and Guiding Treatment

An automated gold nanoparticle bio-barcode assay probe has been described for the detection of PSA at 330 fg/mL, along with the results of a clinical pilot study designed to assess the ability of the assay to detect PSA in the serum of 18 men who have undergone radical prostatectomy for prostate cancer (Thaxton et al. 2009). Available PSA immunoassays are often not capable of detecting PSA in the serum of men after radical prostatectomy. This new bio-barcode PSA assay is approximately 300 times more sensitive than commercial immunoassays and all patients in this study had a measurable serum PSA level after radical prostatectomy. Because the patient outcome depends on the level of PSA, this ultrasensitive assay enables (1) informing patients, who have undetectable PSA levels with conventional assays but detectable and nonrising levels with the barcode assay, that their cancer will not

recur; (2) earlier detection of recurrence because of the ability to measure increasing levels of PSA before conventional tools can make such assignments; and (3) use of PSA levels, which would otherwise not be detectable with conventional assays, to follow the response of patients to treatment.

16.21.13.5 Effects of Lifestyle Changes Shown by Gene Expression Studies

Epidemiological and prospective studies indicate that comprehensive lifestyle changes may modify the progression of prostate cancer. A pilot study was conducted to examine changes in prostate gene expression in a unique population of men with low-risk prostate cancer who declined immediate surgery, hormonal therapy, or radiation and participated in an intensive nutrition and lifestyle intervention while undergoing careful surveillance for tumor progression (Ornish et al. 2008). Consistent with previous studies, significant improvements in weight, abdominal obesity, blood pressure, and lipid profile were observed. Gene expression profiles were obtained from RNA samples from control prostate needle biopsy taken before intervention to RNA from the same patient's 3-month postintervention biopsy. Quantitative real-time PCR was used to validate array observations for selected transcripts. Two-class paired analysis of global gene expression using significance analysis of microarrays detected 48 upregulated and 453 downregulated transcripts after the intervention. Pathway analysis identified significant modulation of biological processes that have critical roles in tumorigenesis, including protein metabolism and modification, intracellular protein traffic, and protein phosphorylation. Intensive nutrition and lifestyle changes may modulate gene expression in the prostate. Understanding the prostate molecular response to comprehensive lifestyle changes may strengthen efforts to develop effective prevention and treatment. The study not only provides insights into potential drug targets but also suggests that lifestyle changes could produce benefits akin to therapeutic interventions. Larger clinical trials are warranted to confirm the results of this pilot study.

16.21.13.6 Personalized Peptide Vaccine for Prostate Cancer

HER2/neu protein is also expressed in prostate cancer. High-risk prostate cancer (HRPC) patients demonstrating varying levels of HER2/neu expression have vaccinated with E75 peptide plus GM-CSF to prevent postprostatectomy PSA and clinical recurrences. In a prospective study HER2/neu (E75) vaccine was shown to prevent or delay recurrences in HRPC patients if completed before PSA recurrence (Gates et al. 2009). A phase I clinical trial of Ii-Key/HER-2/neu hybrid peptide vaccine with recombinant GM-CSF as adjuvant in patients with HER-2/neu-positive prostate cancer showed that the vaccine is safe and can induce HER-2/neu-specific cellular immune responses in patients with castrate-sensitive and castrate-resistant prostate cancer (Perez et al. 2010).

A randomized phase II trial of personalized peptide vaccine plus low-dose estramustine phosphate (EMP) versus standard-dose EMP has been conducted in patients with castration-resistant prostate cancer (Noguchi et al. 2010). The combined therapy was well tolerated with increased levels of IgG and cytotoxic T-cell responses to the vaccinated peptides and resulted in an improvement of progression-free survival as compared to the standard-dose EMP alone.

16.22 Future of Personalized Cancer Therapy

There are now unprecedented opportunities for the development of improved drugs for cancer treatment. Most of the genes in the majority of common human cancers are expected to be defined over the next 5 years. This will provide the opportunity to develop a range of drugs targeted to the precise molecular abnormalities that drive various human cancers and will open up the possibility of personalized therapies targeted to the molecular pathology and genomics of individual patients and their malignancies. The new molecular therapies should be more effective and have less severe side effects than cytotoxic agents. To develop the new generation of molecular cancer therapeutics as rapidly as possible, it is essential to harness the power of a range of new technologies. These include genomic and proteomic methodologies (particularly gene expression microarrays); robotic high-throughput screening of diverse compound collections, together with in silico and fragment-based screening techniques; nanobiotechnology; new structural biology methods for rational drug design (especially high-throughput X-ray crystallography and NMR); and advanced chemical technologies, including combinatorial and parallel synthesis.

16.22.1 Challenges for Developing Personalized Cancer Therapies

Two major challenges to cancer drug discovery are (1) the ability to convert potent and selective lead compounds with activity by the desired mechanism on tumor cells in culture into agents with robust, drug-like properties, particularly in terms of pharmacokinetic and metabolic properties, and (2) the development of validated pharmacodynamic endpoints and molecular markers of drug response, ideally using noninvasive imaging technologies.

There is a need for better description of the genetic damage that drives human cancers; this will form the basis for all future studies of cancer in the laboratory and the clinic and will provide immediate benefit for molecular diagnosis of human cancers as a basis for the development of personalized treatment of cancer. Some of the organizations and projects relevant to development of personalized cancer management are described briefly in the following pages.

16.22.2 COLTHERES Consortium

COLTHERES (Colon Cancer and Therapeutics) is a consortium of EU-clinical centers and translational researchers who have received a total of €6.5 million of core funding from the FP7 organization to define and perform biomarker-driven clinical trials to improve colon cancer therapy outcomes. It is a 4-year program that will use comprehensive molecularly annotated colon cancers to define specific biomarkers of response or resistance to signaling pathway agents. The consortium is open to any pharmaceutical developer who wishes to determine which patients are most likely to respond to their novel cancer therapy and perform rapid proof-of-concept clinical trials. It is expected that the program will generate up to 100 new X-MANTM (gene X-Mutant And Normal) genetically defined human cell lines, accurately incorporating key biomarkers that are predicted to cause resistance to new targeted therapies. These cell lines will be owned by Horizon Discovery Ltd., which forms part of the company's strategy to generate at least 2,500 new X-MAN models in 5 years. These models will support drug discovery researchers to understand how complex genetic diseases manifest themselves in real patients and help rationalize many aspects of drug development and therefore the cost of bringing to market new personalized therapies.

16.22.3 Computer and Imaging Technologies for Personalizing Cancer Treatment

In 2008, the Cancer Institute of New Jersey and IBM started collaboration to develop more accurate diagnostic tools aimed at improving cancer treatments and outcomes. They will use advanced computer and imaging technology to create a database where physicians and scientists can compare patients' tissues with digitally archived cancerous tissues for which genomic and proteomic data is available. This will not only lead to more personalized treatment but will also enhance cell and radiological cancer studies. The initiative, funded by a $2.5 million grant from the NIH, is an extension of the 2006 "Help Defeat Cancer" campaign. For that project, researchers used IBM's World Community Grid—a virtual supercomputer based on unused computer time donated by volunteers—to create an expression signature library for breast, colon, head, and neck cancers and to develop reliable analytical tools for high-throughput tissue microarrays. In the next phase, the project will expand into other types of cancer and also create a Center for High-Throughput Data Analysis for Cancer Research. The Center will rely on pattern recognition algorithms for developing diagnostic tools based on archived cancer specimens and radiology images. That information will be integrated with proteomic and genomic data to aid treatment recommendations. Several other institutions, including Rutgers University, Arizona State University, Ohio State University, and the University of Pennsylvania,

are involved in the project. IBM has donated high-performance P6 570 series class systems to the Center, which uses grid technology that allows collaborators from around the country access the Center's database and software.

16.22.4 Genomic Cancer Care Alliance

Genomic Cancer Care Alliance—which currently involves founding organizations Fox Chase Cancer Center, Scripps Genomic Medicine, Omicia, El Camino Hospital, and the Translational Genomics Research Institute—launched a pilot study in 2010 to investigate the ability of WGS to guide treatment for patients who have responded poorly to initial therapy. The alliance is primarily funded by Life Technologies at present and will use the company's SOLiD 4 sequencing platform.

16.22.5 Global Cancer Genomics Consortium

Global Cancer Genomics Consortium (GCGC) is an international collaborative platform that amalgamates cancer biologists, cutting-edge genomics, and high-throughput expertise with medical oncologists and surgical oncologists; they address the most important translational aspects of cancer research and treatment. The annual GCGC symposium was held at the Advanced Centre for Treatment Research and Education in Cancer, Mumbai, India, in 2011 (The Global Cancer Genomics Consortium 2012). The topics included international NGS efforts that explore cancer-specific transcriptomic changes, SNPs, and CNVs in various types of cancers, as well as the structural genomic approach to develop new therapeutic targets and chemical probes. The wide spectrum of studies presented at the symposium indicates that the translation of emerging cancer genomics knowledge into clinical applications can only be achieved through the integration of multidisciplinary expertise. In summary, the GCGC symposium provided practical knowledge on structural and cancer genomic approaches, as well as an exclusive platform for focused cancer genomics endeavors that will be important for the development of personalized medicine for cancer.

16.22.6 Integrated Genome-Wide Analysis of Cancer for Personalized Therapy

An integrated genome-wide analysis of CNV in breast cancer and CRC using approaches that can reliably detect homozygous deletions and amplifications such as SNP analysis and digital karyotyping has revealed that the number of genes altered by major CNVs, deletion of all copies, or amplification is at least a dozen

copies per cell (Leary et al. 2008). This study has identified genes and cellular pathways affected by both CNVs and point alterations. Pathways enriched for genetic alterations included those controlling cell adhesion, intracellular signaling, DNA topological change, and cell cycle control. A comprehensive picture of genetic alterations in human cancer should therefore include the integration of sequence-based alterations together with copy number gains and losses. Combining copy number and sequence data also holds promise for determining whether particular point mutations have a functional effect, the researchers noted. For example, if a gene turns up with a deletion in one sample and a point mutation in another, it could indicate that that point mutation is inactivating. Incorporating information on other genome-wide changes such as translocations and epigenetic changes could provide even greater insight into cancer, as will trying to determine the timing with which genetic alterations occur in cells. These analyses could prove useful for cancer personalizing diagnosis and therapy. For example, two-thirds of the breast and colorectal samples tested in the study contain alterations to four key signaling pathways, suggesting that drugs targeting these pathways could prove useful for treating both breast cancer and CRC. Since several breast cancer samples tested contained changes to DNA topological pathways, some of these tumors may be candidates for topoisomerase-targeted therapies.

16.22.7 International Cancer Genome Consortium

In 2008, research organizations from around the world launched the International Cancer Genome Consortium (ICGC), which will have an impact on personalized management of cancer. ICGC aims to generate high-quality genomic data on up to 50 types of cancer through efforts projected to take up to a decade. The web site (http://www.icgc.org/) displays ICGC White Paper, detailing its policies and guidelines. ICGC invites research organizations in all nations. Current ICGC members include:

- Australia: National Health and Medical Research Council (Observer Status)
- Canada: Genome Canada; Ontario Institute for Cancer Research
- China: Chinese Cancer Genome Consortium
- Europe: European Commission (Observer Status)
- France: Institut National du Cancer
- India: Department of Biotechnology, Ministry of Science & Technology
- Japan: RIKEN; National Cancer Center
- Singapore: Genome Institute of Singapore
- United Kingdom: the Wellcome Trust; Wellcome Trust Sanger Institute
- United States: NIH

Each ICGC member intends to conduct a comprehensive, high-resolution analysis of the full range of genomic changes in at least one specific type or subtype of cancer, with studies built around common standards of data collection and analysis. Each project is expected to involve specimens from 500 patients and have an

estimated cost of $20 million. As part of its coordination efforts, the ICGC will generate a list of 50 cancer types and subtypes that are of clinical significance around the globe. ICGC members plan to assume responsibility for specific cancers, and one of the ICGC's roles would be to facilitate the exchange of information to avoid duplication of participants' efforts. The ICGC's main criteria for prioritizing cancer types include impact, incidence, age of onset, mortality rates, and availability of therapies; scientific interest; and the ability to obtain enough high-quality samples to conduct a large-scale project.

To facilitate comparisons among different types of cancer, the ICGC guidelines list key factors for its members to consider in the production of genomic catalogs. Those factors include comprehensiveness, which involves detecting all cancer-related genetic mutations that occur in at least 3 % of tumor samples; resolution, which involves generating data at the level of individual DNA bases; quality, which involves monitoring based on common standards for pathology and technology; and controls, which involves comparisons of data from matched, noncancerous tissue.

ICGC member nations will agree to common standards for informed consent and ethical oversight. Although the informed consent process will necessarily differ according to each member country's requirements, the consortium's policies state that cancer patients enrolled in an ICGC-related study should be informed that their participation is voluntary, that their clinical care will not be affected by their participation, and that data obtained from analyses using their samples will be made available to the international research community. ICGC members also should take steps to ensure that all samples will be coded and stored in ways that protect the identities of the participants. To maximize the public benefit from ICGC member research, data will be made rapidly available to qualified investigators. All consortium participants agree not to file any patent applications or make intellectual property claims on primary data from ICGC projects.

16.22.8 PREDICT Consortium

PREDICT (Personalised RNA Interference to Enhance the Delivery of Individualised Cytotoxic and Targeted Therapeutics Consortium) was created in 2009 to coordinate single-drug clinical trials with personalized tumor functional genomic analysis to define patient-specific drug sensitivity pathways and biomarkers predictive of drug response. Partners in the consortium include Horizon Discovery Ltd., Technical University of Denmark, Cancer Research UK, the Wellcome Trust Sanger Institute, Institut Gustave Roussy, The Royal Marsden NHS Trust, and Bayer HealthCare.

The consortium integrates expertise in renal carcinoma clinical trial recruitment, WGS technologies, ex vivo cancer cell line cultures, and personalized RNAi screening technologies. Research is supported by a grant that Horizon Discovery is sharing with the University of Torino Medical School to develop models of inherited and somatic genetic variation for research into new drugs and diagnostics for cancer.

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K.K. Jain, *Applications of Biotechnology in Oncology,* DOI 10.1007/978-1-4614-9245-0,
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