Gabriel D. Dakubo

Cancer Biomarkers in Body Fluids

Biomarkers in Circulation

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To my best friend and wife, Crescentia, and our beloved children, Collins, Ethan, Bernard, and Zaneta.

In memory of George and Nicholas.

Preface

Cancer is a complex disease that continues to burden global healthcare. This affliction can mostly be safely curtailed with enormous investments in technology, coupled with the required regulatory oversight, especially in regard to the biomarker and drug development and approval processes. Efforts at the regulatory and other institutional levels are accelerating the pace of the cancer biomarker development process. Thus, a new era of cancer care has dawned due to the important utilization of cancer biomarkers in companion diagnostics and personalized biotherapies.

The early detection of cancer enables the successful deployment of curative-intent interventions including surgery and chemoprevention. Detecting all cancers at their precursor stages is a formidable task, such that currently only a few cancers are adequately screened for at the population level (e.g., Pap smear). Several strategies target risk group identification for surveillance, employing approaches such as invasive endoscopies and imaging. Although these "screening" procedures are important to cancer management, they are not suitable for population-wide screening programs for early detection of cancer, because of the associated prohibitive costs, radiation exposures, and invasiveness, with their associated possible complications. Visualization of the molecular alterations that drive cancer initiation, progression, and therapy response are becoming possible with molecular imaging. However, until the development of modalities or devices that are safe, effective, affordable, less complicated, and easy to use at the point-of-care or community level, molecular imaging is unlikely to become a routine screening tool for cancer early detection in all communities. Especially deprived by such screening devices will be the resource-poor parts of the world where unfortunately most cancers (57 % in 2012) are diagnosed, and also where many cancer-related deaths (65 % in 2012) occur.

Body fluids obtained by noninvasive or acceptable minimally invasive procedures are a rich source of biomarker information that is suitable for cancer screening and monitoring. Technological advancements, such as single-cell analysis, as well as chip and microfluidic technologies, are paving the way for the easy capture

of cancer biomarkers in almost all body fluids. Coupled with nanoscale device manufacturing, it is now "practically" possible to accurately detect altered cancer targets using low-cost and easy-to-use portable devices at the point of care. The future desires such care, and cancer biomarkers in circulation will immensely contribute toward fulfilling this void. Achieving this feat requires validated cancer biomarkers, which are currently limited. "*Biomarkers in body fluids*" provides a landscape of what has been accomplished in this direction.

To make it an easy read and reference text, each chapter is organized in a similar defined pattern. The relevant molecular pathology of each cancer is provided, followed by circulating biomarkers. The sections on circulating biomarkers explore their presence in the epigenome, genome, transcriptome (coding and noncoding), proteome, and metabolome. The biomarker potential and available clinical applications of circulating tumor cells and tumor-derived extracellular vesicles conclude each chapter.

I am grateful to my project coordinators, Ursula Gramm and Martina Himberger, for seeing this project through to completion, and to my production team, Daniel Ignatius Jagadisan and R.R. Pavan Kumar, for their diligent and expedited work. To my family, "finally it is your turn. . .this one is for you," although it cannot recover the lost family time.

Thunder Bay, Ontario, Canada Gabriel D. Dakubo
August 2016

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Dr. Gabriel D. Dakubo is an Assistant Professor of Molecular Medicine and Human Anatomy in the Division of Medical Sciences at the Northern Ontario School of Medicine. He received his BSc and MBChB degrees from the University of Ghana, followed by a Postdoctoral research fellowship in Molecular Medicine at the Ottawa Hospital Research Institute, Canada. Dr. Dakubo's passion is in noninvasive deployment of biomarkers for cancer management. While an expert in mitochondrial genetic alterations in cancer, he also has a keen interest in the inter-genomic communications that occur in the cancer cell, as well as the Slaughter's concept of field cancerization. Dr. Dakubo is well published and is a reviewer of a number of esteemed journals, including Clinical Cancer Research.

Chapter 1
Melanoma Biomarkers in Circulation

Key Topics

- Molecular pathology of melanoma
- Circulating melanoma cell-free nucleic acid biomarkers
- Circulating melanoma epigenetic biomarkers
- Circulating melanoma genetic biomarkers
- Circulating melanoma protein biomarkers
- Circulating melanoma cells

Key Points

- The molecular pathology of melanoma is well established to include promoter methylations, mutations, and loss of heterozygosity (LOH) of key genes (e.g., *BRAF*) that alter signaling pathways including the MAPK, PI3K, and cell cycle control. The ability to detect and measure these molecular changes in circulating tumor DNA provides real-time noninvasive means of patient management.
- A number of proteins show differential circulating levels in melanoma patients. Although not specific to melanoma, their utility in disease management, such as prognostic and treatment predictions, is clinically informative.
- Melanoma is a highly metastatic cancer that spreads mostly via lymphatics to sentinel lymph nodes (cutaneous) and through the blood stream (uveal). Advanced stage disease is associated with the shedding of cancer cells into the circulation, the detection of which offers prognostic and treatment prediction applications in disease management.

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1.1 Introduction

Melanoma accounts for about 4 % of all skin cancers, but is the most aggressive form, being responsible for >70 % of deaths from cutaneous tumors. The global incidence is rising more rapidly than any other forms of cancer, with an estimated doubling in incidence every couple of decades. The 2012 global incidence, mortality, and 5-year prevalence rates are 232,130, 55,489, and 869,754, respectively. The 2016 estimated incidence and mortality for the US stand at 76,380 and 10,130, respectively. The high prevalence is partly attributed to increased patient survival due to early detection and improved management, especially in the more developed world where >80 % of all cases are diagnosed.

There are multiple predisposing risk factors of melanomagenesis. Sun exposure (UV skin damage), especially early in life, is an established risk factor. People with fair skin, blond or red hair, blue eyes, and who are unable to tan properly, or who sunburn or freckle easily have elevated risk above the general population. Other risk factors include the presence of precursor lesions such as atypical moles (dysplastic nevi) and congenital melanocytic nevus. About 10 % of people who develop melanoma have family history of the disease. Globally, the highest rates are among Australians and New Zealanders.

Early stage lesions are curable by surgery. Hence, early detection increases the cure rate, because stage I disease is associated with 90 % 5-year survival, but fatality rate increases with stage progression. The 5-year survival rate is 60 % for stage II, 10 % for stage III, and very dismal for stage IV. Late diagnosis, early distant organ invasion, and lack of efficacious therapeutic approaches for metastatic disease are some of the reasons responsible for the increased mortality from melanoma.

Clinical features of melanoma are quite distinct, making early detection relatively easy, if screening were established. While diagnosis may easily be established with skin examination, complemented by the use of modern diagnostic techniques such as whole-body photography, dermoscopy, and in vivo confocal microscopy, disease biology can be unpredictable, thus necessitating the need for evidence-based objective biomarkers for clinical management of melanoma. Advances made in the study of melanoma molecular genetics, coupled with current available targeted biotherapeutic agents, should improve the future outlook for patients with this disease. Biomarkers that can be assayed serially and noninvasively for assessing disease progression, prognosis, therapy selection, and monitoring, as well as for early detection of recurrences, should improve clinical management of melanoma.

Melanoma is a known solid tumor with the highest metastatic potential. Vertical-phase melanoma spreads and metastasizes via the circulation (lymphatic and blood) to sentinel and regional lymph nodes, as well as visceral organs including the lungs, liver, brain, bone, and the gastrointestinal tract. Because of the high propensity to spread, melanoma biomarkers are abundant in the circulation and are usually

elevated at a much higher level in advanced than early stage disease. These biomarkers are measurable for clinical applications.

1.2 Screening Recommendations for Melanoma

Based on insufficient evidence of benefit vs. harm, the US preventive services task force does not recommend routine skin examination for the early detection of melanoma in the general adult population. However, skin examination is necessary as a surveillance for people with a family history of melanoma in two or more blood relatives, as well as individuals with multiple atypical moles (dysplastic nevi) or actinic keratosis. In this high-risk population, whole-body photography is periodically performed to screen for suspicious lesions.

1.3 Molecular Pathology of Melanoma

The majority (~90 %) of melanomas are cutaneous, with the remaining being uveal (ocular), mucosal, or leptomeningeal. Cutaneous melanomas originate from neural crest-derived melanocytes in the epidermis or very occasionally in the dermis. The molecular pathology of melanoma is fairly well characterized. Alterations in specific molecules that control oncogenic signaling pathways are implicated in this disease, and hence targeted therapies are at various stages of clinical trials. Established are alterations in the MAPK and PI3K pathways, as well as RB/TP53 and cell cycle control in melanoma progression. In fact, a number of targeted agents, specifically vemurafenib and dabrafenib (BRAF targets) and trametinib (MEK target), are clinically available. This session provides a synopsis of genes altered in melanoma that influence the designated signaling pathways.

1.3.1 BRAF *Mutations in Melanoma*

The *BRAF* proto-oncogene belongs to the RAF family of serine/threonine kinases. Other members are *ARAF* and *CRAF* (RAF1). All activate the MAPK pathway, but their phosphorylation targets and effects are different. *BRAF* is mutated in ~90 % of human cancers. It is commonly mutated in melanoma and papillary thyroid carcinoma (at ~69 % frequency). Somatic missense mutations that affect the kinase domain are found in ~66 % of malignant melanomas. Specifically, the T to A substitution in exon 11 at codon 600 (V600E) is the most frequent mutation occurring in over 80 % of melanomas. The V600E mutation confers over tenfold kinase activity than the wild-type allele. *BRAF* mutations are early events in melanoma development, being observed in ~82 % of benign nevi. However,

BRAF mutations alone are insufficient to initiate tumor formation. Body fluid analysis for *BRAF* mutations as diagnostic, prognostic, and therapy monitoring for targeted therapy (e.g., with PLX4032 and GSK2118436) has been explored with promising clinical applications.

1.3.2 RAS *Mutations in Melanoma*

The *RAS* gene family includes *KRAS*, *HRAS*, and *NRAS*. *KRAS* is most commonly mutated in many cancers, but *NRAS* mutations are mostly associated with melanoma. Activating mutations, commonly at codons 12, 13, and 61, are found in 15–22 % of cutaneous melanomas. These mutations lead to constitutive activation of *RAS*. While not sufficient to initiate melanoma development, *RAS* mutations are important in maintaining the malignant phenotype, because loss of activated *RAS* causes tumor regression. *NRAS* and *BRAF* mutations are mutually exclusive in melanomas, possibly due to functional redundancy. In very rare occasions, the *NRAS* Q61R mutation is found with $BRAF^{V600E}$ in the same melanoma sample. Similarly, *HRAS* and *KRAS* mutations are found in ~1 % and ~2 % of melanomas, respectively.

1.3.3 Alterations in Cell Cycle Control Genes in Melanoma

Loss of cell cycle control is a common feature of almost all malignancies. The cell cycle has positive and negative regulators that function to restrain unwanted cell growth. The cyclin-dependent kinase (CDK) inhibitors (e.g., p16 and p21) are negative regulators, while CDKs drive cell cycle progression by interacting with specific cyclins. Cyclin D1/CDK4, cyclin D1/CDK6, and cyclin E/CDK2 complexes control the G1-S transition phase, where DNA synthesis occurs and the cell is committed to progress through the cycle. The RB protein is the gatekeeper of this transition phase. In non-proliferating cells, RB sequesters E2F, preventing its translocation into the nucleus and induction of genes needed for transition from G1 to S phase. This control is made possible because the cyclin/CDK complexes are inhibited when they bind to the 16 kDa inhibitor of CDK4A (p16INK4A, also known as cyclin-dependent kinase inhibitor 2A (CDKN2A) or major tumor suppressor). In proliferating cells such as the cancer cell, this control is abolished, and hence, cyclin/CDK complexes phosphorylate and inactivate RB leading to the release of E2F transcription factor to induce expression of genes that mediate cell cycle progression.

In familial atypical multiple mole melanoma where there is elevated risk of melanoma and pancreatic cancer, germline *CDKN2A*/*INK4A* mutations are found in as many as 40 % of cases. In sporadic melanomas, several alterations in cell cycle genes are also reported. *CDKN2A*/*INK4A* is silenced, probably through promoter

hypermethylation in as many as 75 % of melanomas. *CCND1* amplifications are observed, so are amplifications in *CDK4* that are commonly found in acral and mucosal melanomas. *CDK4* amplification is however dispensable (not observed) in melanomas with loss of both *CDKN2A*/*INK4A* alleles. Activating *CDK4* mutation (R24C) interferes with p16 binding, but not cyclin D1, leading to constitutive cyclin D/CDK4 activity and cell cycle progression. CDK6 overexpression has also been reported in melanomas.

1.3.4 TP53 *Mutations in Melanoma*

As the guardian of the genome, *TP53* tumor suppressor gene maintains genomic integrity by responding to cellular stress such as exposure of the skin to UV radiation. Being a transcription factor, p53 responds to cellular stress by initiating expression of a set of genes involved in DNA repair, cell cycle arrest, and apoptosis. There are multiple regulatory circuitries for *TP53*; however, a well-characterized regulator of p53 is *MDM2*. In cancerous cells, MDM2 binds to p53 and targets it for ubiquitin-mediated proteasomal degradation. Another safeguard for the cell is stabilization of p53 by p14/ARF, an alternate spliced translated product from the *CDKN2A* locus on chromosome 9p21. The p14/ARF complexes with MDM2 and hence frees p53. *TP53* and *CDKN2A*/*ARF* mutations are rare in melanoma. Mutations in *CDKN2A*/*ARF* are found in ~2 % of melanomas.

1.3.5 KIT *Mutations in Melanoma*

KIT is a 109.865 kDa receptor tyrosine kinase encoded by *KIT* (CD117) located on chromosome 4q12. Following receptor ligand (stem cell factor of kit ligand) interaction, a number of signaling pathways are modulated. These include the MAPK, PI3K, and phospholipase C pathways. Additionally KIT targets the induction of genes such as *MITF* and *Src*. KIT is involved in melanocyte survival, proliferation, differentiation, and migration and melanin production. Mutations lead to lack of melanoblast migration and loss of KIT-negative melanoblasts. C-KIT is activated in many malignancies including melanoma. Work by Curtin and colleagues revealed that *C-KIT* mutations or increase in copy number occurs in 39 % of mucosal, 36 % of acral, and 20 % of chronically sun-damaged skin melanomas but not in non-chronically sun-damaged skin melanomas [1]. Mutations in *KIT* include K642E (common in gastrointestinal stromal tumors) and N566D. The genetic alterations in *KIT* are associated with increased expression and constitutive tyrosine kinase receptor activation.

1.3.6 MITF *Mutations in Melanoma*

MITF is a basic helix-loop-helix leucine zipper transcription factor on chromosome 3p14.2-p14.1 that belongs to the MiT family of transcription factors. The members form homodimers and heterodimers. MITF-M isoform regulates melanomagenesis because of the presence of a melanoma-restricted promoter in this isoform. MITF-M usually induces the transcription of genes including tyrosinase (TYR), tyrosinase-related protein (TYRP1), and dopachrome tautomerase (DCT) required for melanin production. Signaling pathways that target MITF include the C-KIT/MAPK, WNT/β-catenin, and α-MSH/cAMP and gp130 pathways. MITF amplifications (up to 100-fold in some cases) and overexpression are found in ~10 % of primary and ~20 % of metastatic melanomas. Increased transformation and anchorage independence and poor survival are features of MITF overexpression. MITF G1075A (E318K) mutation causes impaired sumoylation and aberrant regulation of MITF targets leading to increased cellular proliferation, migration, and invasion. This mutation is found in both familial and sporadic melanomas [2].

1.3.7 PI3K Pathway Alterations in Melanoma

The PI3K pathway that controls important cellular processes such as survival, proliferation, invasion, and glucose metabolism is deregulated in several tumors. The AKT3 isoform is overexpressed in melanoma, and phospho-AKT is detected in 54 % of nevi and at a frequency of 71 % in primary and metastatic melanomas [3]. Loss of chromosome 10 (*PTEN* locus) occurs in 30–60 % of sporadic melanomas [4]. Loss of *PTEN* expression is observed in 30–50 % of melanoma cell lines and in 5–20 % of primary melanomas [5]. *PTEN* somatic mutations occur in association with *BRAF* mutations (but not *NRAS*). *PTEN* epigenetic silencing is also demonstrated in melanomas [6].

1.3.8 Molecular Subtypes of Melanoma

Using comparative genomic hybridization (CGH), DNA sequencing, and immunohistochemistry (IHC), Curtin et al. provided a genetic classification of melanoma [7]. Based on skin sites involved and UV exposure, four groups of cutaneous melanoma with defined genetic alterations have been identified. These groups are melanomas associated with chronic sun-induced damage (CSD), without CSD (non-CSD), as well as mucosal and acral melanomas. Chronic sun-induced damage melanomas rarely harbor *BRAF* and *NRAS* mutations. The predominant findings in these tumors are *CCND1* copy number gains. Non-CSD melanomas are associated with *BRAF* (59 %) and *NRAS* (22 %) mutations. These tumors also harbor

chromosome 10 loss (*PTEN* locus) and amplifications in *CDK4* and *CCND1*. Landi et al. provided evidence that *MCIR* variant alleles confer melanoma risk in non-CSD skin, and these melanomas have *BRAF* mutations [8]. Deletions in *CDKN2A* characterize mucosal melanomas, while acral melanomas have increased incidence of *CDK4* amplification, but melanoma cells with homozygous *CDKN2A* deletions lack *CDK4* amplification.

1.3.9 Molecular Progression Model of Melanoma

Melanoma develops in a very defined progressive model proposed by Clark and coworkers in 1984 (Fig. 1.1) [9]. The identified five defined steps include acquired or congenital nevi with normal melanocytes, dysplastic nevi with atypical melanocytes, radial growth phase primary melanoma without metastatic activity, vertical growth phase primary melanoma with metastatic competence, and finally metastatic melanoma. However, this model accounts for ~35 % of melanomas, indicating the presence of alternative pathways of melanoma development. Various epigenetic and genetic alterations drive melanoma progression. The classic Clark pathway involves early alterations in *BRAF* and *NRAS* as observed in nevi, followed by *CDKN2A* and *PTEN* mutations that drive the development of melanoma but in the radial growth phase. The subsequent evolution into vertical growth phase melanoma with invasive propensity requires additional alterations in *CDK2*, *CCND1*, and PI3K pathway, among several others. Finally, the loss of normal

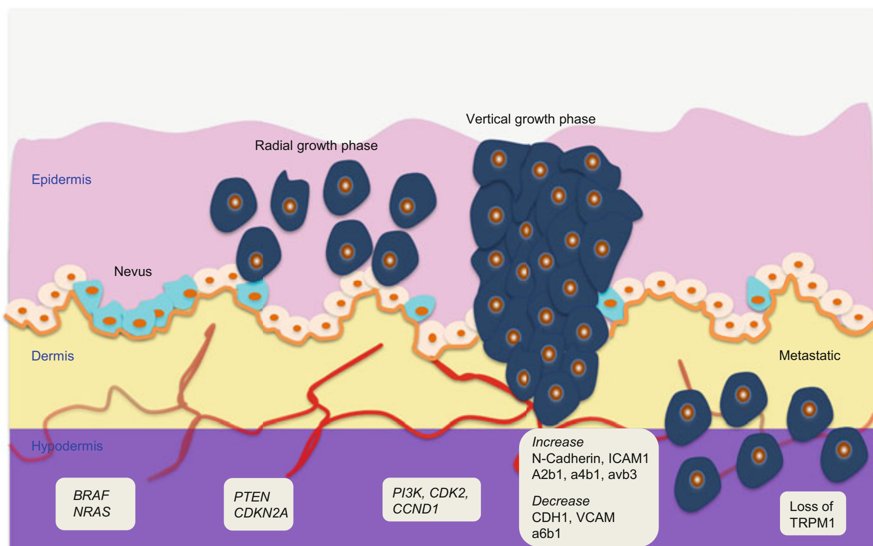

Fig. 1.1 Molecular pathology of multistep melanoma progression model

homeostatic interactions between melanoma cells and the extracellular stromal matrix, keratinocytes, endothelial cells, fibroblasts, and immune cells propel these cells into invasive state. Thus, loss of cell-cell and cell-microenvironmental communications mediate progression. Cellular adhesions play an important role in this process. Alterations in the major groups of adhesion molecule receptors, cadherins, integrins, and cellular adhesion molecules of the immunoglobulin superfamily mediate metastasis.

The molecular pathways of uveal melanoma are not well understood. However, emerging work indicates the involvement of *GNAQ*, a gene that encodes the q class of G protein α-subunit, involved in signaling between G protein-coupled receptors and their downstream components. Mutations in *GNAQ*, especially at codon 209, are common (~46 %) in uveal melanomas, while activating *BRAF* and inactivating *CDKN2A* mutations are mostly absent. Understandably, these *GNAQ* activating mutations are capable of engaging the MAPK pathway (without the need for *BRAF* and *NRAS* mutations) [10].

1.4 Circulating Melanoma Biomarkers

Noninvasive biomarkers for the management of melanoma patients will greatly improve patient outcomes. Thus, the genetic alterations in melanoma tissues are being actively pursued in circulation. Specifically, ctDNA and the associated epigenetic and genetic alterations, as well as altered protein levels have shown promise. Attention has also been given to the clinical importance of circulating melanoma cells.

1.4.1 Circulating Melanoma Cell-Free Nucleic Acid Biomarkers

Studies on circulating cell-free DNA (ccfDNA) in melanoma have primarily focused on qualitative tumor-specific alterations such as analysis of methylation and mutations in genes including *RASSF1A* and *BRAF*. Work by Orlando's group on ccfDNA in melanoma patients had initially indicated that DNA integrity index (DII), defined as the ratio of 180 bp to 67 bp PCR fragments, was a reliable discriminatory biomarker for identifying patients with melanoma [11]. The explanation for this finding is that melanoma and other cancer patients have mostly larger fragmented DNA of sizes 181–307 bp compared to healthy control individuals that harbor mostly shorter apoptotic fragments (67–180 bp). In a follow-up study, this group included measurement of total ccfDNA concentration, and detection of *BRAF*^{V600E} mutation and *RASSF1A* promoter methylation in the analyses. Whereas ccfDNA concentration may not be accurate enough for the detection of many

cancers, this study found that the best performance in detection of melanoma was total ccfDNA concentration with area under the receiver operating characteristic curve (AUROCC) of 0.85, followed by DNA integrity index (AUROCC of 0.79), with the cancer-specific biomarkers performing poorly (*RASSF1A*; AUROCC of 0.69, and *BRAF*^{V600E}; AUROCC of 0.64). The explanation could be that not all tumors will express a specific genetic marker (e.g., not all melanomas harbor *BRAF* mutations). The combination of the three biomarkers (DNA concentration, mutations, and methylation), however, increased the accuracy of melanoma detection (AUROCC of 0.95) [12].

1.4.2 Circulating Melanoma Epigenetic Biomarkers

In melanoma, a CpG island methylator phenotype (CIMP) has been described. There are over 50 genes with promoter hypermethylation in melanoma. An epigenetic control of DNA methylation is exemplified by the regulatory functions of miR-29c. miR-29c controls the expression DNMT3A and DNMT3B, which are upregulated in melanoma progression. This may have effects on the observed hypermethylation of multiple genes, including *WIF1*, *RASSF1A*, *TFP12*, and *SOCS1* in melanoma progression. The tumor suppressor *RASSF1A*, for example, is methylated in ~57 % of melanomas. This gene silencing, and loss of tumor suppressor function, strongly correlates with melanoma progression and prognosis. Similarly, methylations of methylated-in-tumor 17 and methylated-in-tumor 31 (*MINT17* and *MINT31*) are associated with specific tumor gene methylation patterns. Another miRNA, miR-532-5, which is upregulated in metastatic melanoma, controls *RUNX3* expression, and *RUNX3* promoter methylation occurs in a subset of primary and metastatic melanomas.

Genes frequently methylated in melanoma have been examined in serum as biomarkers of disease progression, prognosis, and/or therapy response. Work primarily from Hoon's laboratory has associated methylation of *RASSF1A*, *RARβ2*, *MGMT*, and *ERα* with melanoma prognosis and response to biochemotherapy. Gene promoter methylation in sera as predictive biomarkers in patients on concurrent biochemotherapy has been assessed. Methylation of *RASSF1A*, *RARβ2*, and *MGMT* was assayed in pretreatment sera and related to response to treatment. Responders had significantly less methylated *RASSF1A* (13 %) than nonresponders (42 %). The presence of at least one methylated gene conferred worse survival outcome in patients than those without methylated genes. *RASSF1A* methylation was the only gene alteration significantly associated with overall survival and biochemotherapy response [13]. In another study, promoter hypermethylation of *ERα* appeared to be a biomarker of melanoma progression, being more frequent in advanced metastatic disease than in localized melanoma. The clinical relevance of serum *ERα* methylation in patients on biochemotherapy and tamoxifen was also explored. Consistent with tissue analysis, methylation in serum was associated with advanced stage disease and was the only predictive factor of progression-free

survival (PFS) and overall survival (OS) in patients on biochemotherapy [14]. Koyanagi et al. conducted a study on the predictive value of methylation markers and CTCs in peripheral blood from melanoma patients [15]. CTCs were targeted by amplification of *MART-1*, *GalNACT*, and *MAGEA3* transcripts. CTCs were detected in 86 % of patients with methylated *RASSSF1A* and *RARβ2* DNA compared to 37 % of those without gene methylation. The CTC markers significantly correlated with methylation, and both biomarkers correlated with biochemotherapy outcome. The presence of both methylated DNA and CTCs significantly predicted worse response to biochemotherapy treatment and shorter time to PFS and OS. Methylation status of LINE and absent in melanoma 1 (*AIM1*) were assessed in tissue and sera from melanoma patients. LINE hypomethylation was observed more frequently in sera from patients with primary melanoma, while *AIM1* promoter hypermethylation was associated with metastatic disease. In multivariate analysis, methylation of *AIM1* was a significant predictor of OS. Consistent with tissue alterations, in serum analysis, LINE hypomethylation was more frequent in stage I/II disease than in healthy controls, and *AIM1* promoter hypermethylation was a predictor of OS in stage IV patients [16].

Methylation of selected genes has shown potential for melanoma detection. Methylation of five genes (*SOCS1*, *SOCS2*, *RASSF1A*, *CDKN*, and *MGMT*) was assayed in sera from patients with melanoma, nevi, other skin tumors, metastatic non-cutaneous cancers (breast and colon cancers), chronic inflammatory diseases, and healthy controls. Methylation of all these genes was detected in melanoma patient samples, with frequencies ranging from 43 to 75 %. Eighty three percent (83 %) of the samples had methylation in at least one gene, and as many as 20 % harbored methylation of all five genes, and these methylation profiles were distinct from those in the other tumors examined. In primary melanoma tissue samples, *SOCS2*, *CDKN*, and *RASSF1A* levels were downregulated, while *MGMT* was upregulated 12-fold [17].

1.4.3 Circulating Melanoma Genetic Biomarkers

The mutations in genes as well as microsatellite alterations with disease progression are detectable and measurable in circulating fluids from patients.

1.4.3.1 Mutations as Circulating Melanoma Biomarkers

Specific gene mutations in melanoma have been assayed in circulating blood as noninvasive biomarkers for disease management. Expectedly, *BRAF* mutations dominate the various studies, which primarily have come from David Hoon's group. One study by this group indicates that *BRAF* mutations in serum can be used to predict OS (i.e., mutation is associated with poor outcome) and response to biochemotherapy (responders are often mutation negative). In this initial study,

37 % and 39 % of stage I/II and III/IV disease patients, respectively, were positive for *BRAF* mutations in serum samples, and this was significantly associated with worse OS. Before treatment, *BRAF* mutations were detectable at a frequency of 42 % each in responders and nonresponders. However, following biochemotherapy, the detection rates of *BRAF* mutation were 10 % and 70 % in responders and nonresponders [18]. In another study, the detection rate of $BRAF^{V600E}$ mutation in plasma/serum was obtained in 80 % of patient but not in any control samples [19]. The positive samples were mostly from patients with advanced stage (IV) disease, suggestive of the usefulness of circulating $BRAF^{V600E}$ mutation detection in monitoring advanced stage disease patients.

BRAF mutation analysis in serum DNA may be useful for patient selection for therapy as well. The clinical relevance of *BRAF* mutation analysis in ccfDNA was examined in advanced stage melanoma patients in phase II clinical trial with MEK1/2 inhibitor [20]. *BRAF* mutations were detected in 47.9 % and 26.2 % of tissue and serum samples, respectively. However, the frequency of detection was 55.6 % (higher) in those with tissue mutations. The potential noninvasive use to select patients for treatment is evident from this study [20]. In $BRAF^{V600E/K}$ mutation-positive stage IV patients in phase II trial with BRAF inhibitors, dabrafenib, clinical outcome prediction was evaluated by comparing *BRAF* mutation status in ctDNA and tissue samples, as well as ccfDNA levels. Baseline ccfDNA levels predicted PFS in patients with $BRAF^{V600E}$ mutations in this trial. This finding shows potential for monitoring treatment response [21]. A sensitive detection method (able to detect 3:100 mutant alleles) was used to quantify $BRAF^{V600E}$ mutation in plasma from patients with melanoma and non-melanoma skin cancer. The performance of this assay using absolute concentrations of *BRAF* mutations was diagnostic at a sensitivity of 97 % and a specificity of 83 %. *BRAF* mutations in plasma were concordant at 80 % with tissue mutations [22]. This assay should augment the translational potential of *BRAF* mutation detection in circulation.

Mutation analyses in circulation have also been performed in patients with uveal melanoma. Circulating tumor cells and gene mutations in ccfDNA were assessed in samples from patients with metastatic uveal melanoma. *GNAQ* c.626 A > T, c.626 A > C, and *GNA11* c.626 A > T copy numbers were quantified in plasma, and CTCs were assayed using CELLSEARCH® technology. CTCs were detected at a rate of 30 % compared to ccfDNA using gene mutations, which were found in 84 % of cases. Circulating tumor DNA concentration and CTCs were associated with hepatic spread, metastatic volume, as well as PFS and OS. In multivariate analysis, ctDNA was superior to CTCs as a prognostic biomarker [23]. Activating mutations in exon 4 (R183) and exon 5 (Q209) of *GNAQ* and *GNA11* are almost exclusive to uveal melanoma. As a proof of principle, ultra-deep sequencing was used to detect ctDNA targeting these mutations. The Q209 mutation (present in 2–38 % mutant reads) was detected in either *GNAQ* or *GNA11* in 40.9 % of plasma from patients with metastasis. The potential for early detection of micrometastasis needs pursuance [24].

1.4.3.2 Circulating MSA as Melanoma Biomarkers

In addition to *BRAF* mutations, microsatellite alterations (MSA) are detectable in circulation of melanoma patients. Once again, the Hoon's group questioned whether tumor-specific genetic changes could be detected in plasma from melanoma patients, and whether this had any clinical relevance. First, ten microsatellite markers on six chromosomes commonly harboring LOH in melanoma were used to assess alterations in tissue and matched plasma samples from patients with early and advanced stage melanoma. Forty matched tissue and plasma samples were significantly concordant for LOH, which tended to be more frequent in advanced stage disease. Clinical disease progression was significantly associated with plasma LOH at D3S1293, as well as the combination of LOH at D9S157/D3S1293, D9S157/D1S228, and D11S925/D3S1293 [25]. A follow-up study assessed the prognostic value of MSA in preoperative blood samples from melanoma patients. LOH on the six chromosomes was used in this study of patients undergoing surgical resection of melanoma. LOH was present in 56 % of patients and was associated with advanced stage disease. LOH in plasma samples was an independent preoperative indicator of increased risk of death. The microsatellite alterations at D1S228 significantly correlated with poor survival after surgery [26]. Microsatellite analysis as a measure of allelic instability as well as mutations in specific genes has been explored in the circulation as prognostic biomarkers of melanoma. Nine microsatellite markers were used for analyses of sera from stage IV melanoma patients. The findings indicated that allelic instability at these sites was associated with a much lower response to therapy and independently predicted disease progression [27]. Because allelic instability at the *APAF-1* locus (12q22–23) is more frequently found in metastatic melanoma, serum as a noninvasive sample was assayed for possible use in prognostic and therapy response monitoring. Instability at this locus is significantly higher in patients who do not respond well to treatment and is also associated with poor survival [28]. Takagi et al. targeted detection of recurrence of mucosal melanoma after radiotherapy by analyzing LOH in plasma samples [29]. Pretreatment samples were interrogated using 4 (D1S243, D6S311, D9S161, D19S246) out of 20 known markers of mucosal melanoma. All markers demonstrated LOH, but the highest frequency was at D9S161 (65 % of plasma samples). LOH in plasma DNA at one or more loci was associated with recurrence and metastasis, and LOH at two loci tended to relate to larger mean tumor volume. Tagagi's group performed a follow-up study using the same markers. Using triplicated whole genome amplification and triplicated PCR, alterations were detected on at least one locus in 70.6 % of samples, and this was associated with metastasis and recurrence. Importantly, LOH in plasma using these markers (D1S243, D6S311, D9S161, and D19S246) strongly correlated with melanoma, and hence could serve as noninvasive screening biomarkers of mucosal melanoma [30].

1.4.4 Circulating Melanoma Coding RNA Biomarkers

Altered gene transcripts, primarily tyrosinase (*TYR*) mRNA, have been targeted in circulation of melanoma patients. As a proof of principle that extracellular mRNA integrity is stable and can be amplified, serum from six patients with malignant melanoma and 20 controls were subjected to tyrosinase mRNA analysis. Four cancer patient samples and none of the 20 control samples were positive for this marker. Even sera frozen for several years could still be assayed for mRNA detection [31]. CTCs are commonly detected in melanoma patients by targeting tyrosinase mRNA. Because CTCs are rare, and that melanoma cells are transient in circulation, molecular CTC assay data have been inconsistent depending on whether isolated cells or whole blood is used for analysis. To address this problem, Hasselmann et al. targeted melanoma-specific mRNA in ccfRNA in serum/plasma samples in comparison to data using blood cell samples from stage IV melanoma patients [32]. In the small samples analyzed, tyrosinase mRNA was detected in 60 % of serum ccfRNA samples compared to 100 % of the blood cell samples [32]. Thus, ccfRNA only samples released *TYR* mRNA from other sources, with possible exclusion of CTCs.

1.4.5 Circulating Melanoma Noncoding RNA Biomarkers

MicroRNA deregulation was initially reported in three primary melanoma samples and two melanoma cell lines included in the analysis by Lu et al. [33]. Zhang et al. included 45 primary cultured melanoma cells in their analysis of miRNA copy number variations [34]. In this series, 85.9 % of the genomic loci harboring the 283 miRNAs examined had copy number abnormalities in melanoma samples. In the NCI-60 panel of cell lines including melanoma cell lines, 15 miRNAs were significantly differentially expressed between melanoma and normal, as well as other non-cutaneous cancer cell lines [35]. However, the first well-designed study of miRNA alterations in melanoma was by Mueller et al. [36]. They profiled the miRNAome of melanoma, normal human melanocytes, and melanoma cell lines from primary and metastatic melanoma. MicroRNA deregulation could be associated with disease progression from early to metastatic stages. The data was also validated by RT-PCR and in primary melanoma tissue samples.

The targets and mode of regulation of some deregulated miRNA in melanoma are provided in some studies. Several relevant tumor-associated genes (*NRAS*, *MITF*, *C-KIT*, *TFAP2*, *CDKN1B*, *RUNX3*, *ITGB3*, *CCND1*, *MET*, *MNT*, *PLZF*, and *FOXO3*) are controlled by miRNAs in melanoma progression. Similarly, some tumor-associated genes act by targeting and regulating miRNA in melanoma. For example, MITF regulates a number of miRNAs including let-7 family, miR-29, miR-125b, miR-146a, miR-148b, as well as miR-221/222, miR-17-92, and miR-106-363 clusters. *RAS* oncogene is a target of let-7 in many cancers. In

melanoma, let-7b directly and indirectly targets genes involved in cell cycle control [37]. Let-7b interacts with the 3′ UTR of *CCND1*, interfering with cell cycle progression. Consistently, some members of let-7, including let-7a, are significantly downregulated in melanoma. Let-7a controls later stages of melanoma progression by regulating integrin β3 expression through interaction with its 3′ UTR. This repression is lost with increasing integrin expression associated with melanoma invasion and metastasis. Additionally, let-7a controls *RAS* oncogene expression in melanoma [38].

Using database search for miRNAs located on chromosome 1p22 that is characterized by melanoma susceptibility genes, Bemis et al. identified miR-137 to be of importance in melanoma [39]. Further computational work showed that *MITF* was a target of this miRNA and that it directly binds and suppresses *MITF* expression. Amplification of variable number tandem repeat (VNTR) located on the 5′ UTR of miR-137 primary transcript interferes with the processing of miR-137. VNTR amplification is observed in some *MITF*-expressing melanoma cells. Another miRNA that controls *MITF* is miR-182 [40]. MicroR-182 is overexpressed in melanoma and is associated with invasive and metastatic propensities of melanoma cells through suppression of *MITF* and *FOXO3*.

MicroRNA-221 and miR-222 are transcribed as a bicistron precursor from the X-chromosome. They are overexpressed in a variety of cancers and target genes including *CDKN1B* and *C-KIT* receptor. In melanoma, *C-KIT* downregulation is associated with disease progression, and *p27Kip1*/*CDKN1B* suppression causes increased proliferation via cell cycle deregulation. In normal melanocytes, miR-221/miR-222 cluster is repressed by promonocytic leukemia zinc finger (*PLZF*) that is a transcriptional repressor of the miR-221/miR-222 promoter. Thus, *PLZF* is lost in melanomas overexpressing these miRNAs [41, 42]. It is even suggested that *C-KIT* may be regulated primarily by miRNAs [43]. Promoter methylation and decreased expression of miR-34a are observed in two thirds of melanomas and are a tumor suppressor in uveal melanoma [44]. MicroRNA-34b, miR-34c, and miR-199a* downregulate *MET* oncogene in melanocytes leading to impaired MET-mediated motility. A number of miRNA biomarkers with emerging functional importance in melanomagenesis are found in Tables 1.1 and 1.2.

The clinical relevance of circulating miRNA in melanoma patients has been addressed. Serum levels of miR-221 are significantly elevated in patients with malignant melanoma compared to healthy controls. Levels were much higher in stages I–IV disease patients than those with in situ melanoma and correlated with tumor thickness. The levels decreased following surgical tumor removal and returned to elevated levels following recurrence [45]. Shiiyama et al. evaluated the utility of circulating miRNA in detection of metastatic melanoma [46]. Six miRNAs (miR-9, miR-145, miR-150, miR-155, miR-203, and miR-205) were examined in patients with and without metastatic disease. A panel of all the miRNAs except miR-203 was sensitive enough to discriminate patients with metastasis from those without. Another study focused on circulating miRNA in sera from healthy controls and those with metastatic melanoma. Loss of normal serum miR-29c and miR-324-3p was predictive of metastasis, and this was even

Table 1.1 Some important melanoma oncomirs and their targets

Oncomirs	Targets
let-7	*CDK4, 6*
MiR-15b	*BCL2*
MiR-17-92 cluster	*MYC*
MiR-21	*PTEN*, *STAT3*, *PDCD4*, *TIMP3*
MiR-27a	*PSMA1*
MiR-30b, d	*GALNT7*
MiR-33a	*PIM1*, *CDK6*, *CCND1*
MiR-100	*PLK1*
MiR-137	*MITF*, *EZH2*, *MET*, *YB1*
MiR-145	*TP53*, *MYC*
MiR-149	*GSK3α*
MiR-150	*NOTCH3*, *EGFR2*
MiR-182	*MITF*, *FOXO3*
MiR-195	*WEE1*
MiR-199a-3p	*APOE*, *DNAJA4*
MiR-199a-5p	*SWI/SNF*, *APOE*, *DNAJA4*
MiR-210	*HIF1α*, *ATM*, *FAS*, *TNFR1*
MiR-214	*ITGA3*, *MET*, *TFAP2C*
MiR-221/222	*P27*, *CCND1*
MiR-340	*RAS*, *RAF*, *MAPK*
MiR-424	*HIF1α*, *HIF2α*
MiR-506-514	*HOXB7*, *PBX*
MiR-532-5p	*RUNX3*
MiR-1908	*APOE*, *DNAJA4*

Table 1.2 Some important melanoma tumor suppressormirs and their targets

Tumor suppressormirs	Targets
Let-7a	*NRAS*
MiR-9	*NK-jB*, *SNAI1*
MiR-29	*DNMTS*
MiR-31	*DNMTS*
MiR-126	*ADAM9*, *MMP7*
MiR-155	*SKI*
MiR-193b	*CCND1*
MiR-148	*MITF*
MiR-203	*E2F3*
MiR-205	*E2F1*, *E2F5*
MiR-211	*MITF*, *AP1S2*, *SOX11*, *KFBP5*
MiR-455	*PAX6*, *NEDD9*
MiR-573	*NCAM*

accurate at differentiating melanoma metastasis from colon and renal cancer metastasis, indicating a high level of specificity for melanoma spread [47]. It will be informative to know whether the other remaining altered miRNAs in melanoma tissue samples are measurable in circulation, as well as their clinical relevance.

1.4.6 Circulating Melanoma Protein Biomarkers

Melanoma serum biomarkers have been explored for multiple applications in patient management. Among them, they are used to complement clinical staging (e.g., serum lactate dehydrogenase—LDH), for prognostication (survival predictions of newly diagnosed patients), for monitoring response to various therapeutic interventions, for monitoring stage progression, and for predicting lymph node status. Detailed in this section are established serum biomarkers and the emerging circulating melanoma proteome and peptidome.

1.4.6.1 Serum S100β as Melanoma Biomarker

The S100 family of acidic calcium-binding proteins comprises 21 members encoded by different genes. They are expressed by diverse cell types and have multiple intracellular and extracellular functions. They control intracellular signal transduction processes through inhibition of protein phosphorylation. They also control cellular morphologic and structural changes through modulation of cytoskeletal dynamics, and are involved in cell-cell communication, cell growth, and energy metabolism. Their extracellular functions include macrophage activation, leukocyte chemoattraction, and control of cell proliferation, apoptosis, and p53 functions.

The expression of S100β was initially identified by Gaynor et al. in cultured human melanoma cells, and later confirmed by Nakajima et al. using immunohistochemistry as being present in human malignant melanoma and pigmented nevus [48, 49]. Fagnart et al. were first to report the elevated levels of S100β in sera from cancer patients, among whom 81.8 % were patients with metastatic melanoma [50]. Subsequently, Guo et al. revealed the clinical value of serum S100β measurements in malignant melanoma as it mirrored clinical stages [51]. Using a cutoff value of 0.15 ug/L, sensitivity was 1.3 % for early stage I/II disease, 8.7 % for stage III, but as high as 73.9 % for stage IV disease. In advanced stage disease, a rise or decline in serum S100β was predictive of disease progression or decline, respectively. Since these initial findings, the clinical relevance of serum S100β levels as a biomarker for clinical staging, prognostic, and possible predictive potential for malignant melanoma has been confirmed by several other studies. However, the levels of S100β are elevated in other neoplastic diseases, as well as in liver and renal injury, inflammatory conditions, infections, and also liver metastasis from any primary, which compromises diagnostic specificity.

Prognostic Utility of Serum S100β in Melanoma

The survival rate of patients with malignant melanoma strongly correlates with S100β serum levels. The observed-to-expected death ratio was significantly increased with elevated serum S100β. At levels >0.6 μg/L, a fivefold increase in relative hazard was observed, and prognostication was independent of clinical stage [52]. In other studies, increasing levels of S100β were associated with clinical stage, disease progression, and lack of response to therapy [53, 54].

Abraha et al. measured serum levels in patients and controls [55]. Median concentrations were 0.11 μg/L, 0.24 μg/L, and 0.39 μg/L in patients with stage I/II, III, and IV, respectively, and these levels were significantly higher than in control individuals (0.1 μg/L). Sensitivity and specificity at a cutoff value of 0.2 μg/L were 82 % and 91 %, respectively, for detection of advanced stage disease. The levels also correlated with Breslow tumor depth. The use of serum S100β levels at a cutoff value of 0.22 μg/L and Breslow thickness >4 mm increased the sensitivity and specificity to 91 % and 95 %, respectively, for detection of secondary spread. Indeed, a large study conducted by Martenson et al. demonstrated the prognostic utility of circulating S100β [56]. S100β was measured by luminescent immunoassay (LIA) method in a large cohort of >1000 patients with stage I–III cutaneous melanoma. Clinical disease stage was significantly related to serum levels, with the lowest levels found in stage I and the highest in stage III disease. In multivariate analysis, serum S100β was the strongest predictor of disease-specific survival in stages II and III disease but not in stage I [56]. A meta-analytical review of 22 studies involving 3393 patients was performed to assess the prognostic value of serum S100β in patients with malignant melanoma. Serum levels were significantly associated with poor survival, with a hazard ratio (HR) of 2.23 ($p < 0.0001$). The studies were homogenous in stage I/II disease (1594 patients). In this subgroup, S100β levels still remained a strong prognostic factor (HR 2.28, $p < 0.0001$). Studies using multivariate analysis confirmed serum S100β has prognostic value independent of TNM staging system [57].

As initially reported by Abraha et al. [55], others have confirmed the fact that serum levels increase with disease progression. The mean S100β concentration was determined to be 0.075 μg/L in stage I–III compared to 0.441 μg/L in stage IV disease patients. Median survival was 256 days for patients with levels >0.150 μg/L compared with 561 days for those with normal values [58]. A multiple biomarker (S100β, LDH, MIA, YKL40) study of advanced melanoma (stages IIIB/C and stage IV) patients revealed that S100β, MIA, and LDH levels were significantly much higher in cancer patients than disease-free controls. S100β and MIA had the best diagnostic sensitivity. MIA was a prognostic factor for OS. However, patients with simultaneous increase in serum S100β and MIA had significantly shorter survival period than those with lower levels [59]. Preoperative S100β levels predicted nodal tumor load, and increased levels were associated with shorter disease-free survival (DFS) in stage III melanoma patients [60]. A retrospective prognostic study of the value of S100β and LDH in patients with metastatic melanoma found in multivariate analysis that S100β (but not LDH) and brain metastasis were independent

predictors of OS [61]. S100β is much superior to LDH in prognostication of stage IIIB/C malignant melanoma. Preoperative and postoperative days 1 and 2 S100β levels were all associated with DFS. In multivariate analysis, preoperative day 2 levels were the strongest predictor of DFS (HR 2.55). Preoperative S100β levels are strongest independent predictor of disease-specific survival (HR 2.81). LDH was not prognostic in these series [62].

S100β has also demonstrated the potential to predict melanoma metastasis. A prospective study of serum S100β and LDH in high-risk melanoma patients on adjuvant chemotherapy revealed that clinical course correlated with elevated S100β and LDH levels. S100β levels, however, outperformed LDH in predicting early distant metastasis. However, both failed to predict locoregional metastasis from small tumors [63]. It was noted that S100β levels were very specific for recurrence prediction but not useful in predicting sentinel lymph node tumor status. Additionally, S100β levels were insensitive in detection of early recurrence [64].

Serum S100β as Melanoma Treatment Response Prediction Biomarker
S100β is a potential biomarker for monitoring response to treatment in patients with melanoma. The chitinase-3-like protein 1 (CHI3L1) gene product, YKL40, is a 40 kDa secreted glycoprotein implicated in cancer, development, and inflammatory disease. YKL40 as a prognostic factor was compared to LDH and S100β. All three biomarker levels correlated with disease stage. Therapy response in metastatic stage IV melanoma was significantly associated only with baseline S100β levels. Strong correlation existed with treatment response when S100β levels declined or remained unchanged overtime, and only S100β levels had significant prognostic impact on survival [65]. In a follow-up study by this group, the predictive value of S100β and LDH in stage III melanoma patients on single bevacizumab therapy prior to therapeutic lymph node dissection was evaluated. In this small number of patients ($n = 9$), it appeared that S100β levels could predict treatment response evidenced by tumor necrosis in lymph nodes. This response was associated with decreased S100β levels [66]. The clinical relevance in response prediction and disease monitoring in metastatic melanoma patients on chemoimmunotherapy has also been reported. In this study, the control group was metastatic renal cancer patients on similar therapy. S100β levels were elevated in 81 % of patients prior to treatment and were significantly much higher in non-responders. Patients with serum S100β levels $\geq$1 μg/L were less likely to respond compared to those with normal or moderately elevated levels. After treatment, 55 % of those with stable disease or in remission had levels lower than the cutoff value, compared to only 5 % of those with progressive disease. LDH was not predictive of response [67].

Hauschild et al. performed the first large-scale study of the value of S100β in stage IV disease patients on chemotherapy and/or immunotherapy [68]. They measured the levels prior to, during, and after treatment. Over the 8-week course of therapy, midterm (4 weeks) analyses revealed a rise in S100β levels in 78 % of patients with progressive disease, and at 8 weeks, in 84 % of those with disease

progression had elevated levels. Among the responders, S100β levels were constant or declined in 95 % at 4 weeks and 98 % at 8 weeks of therapy. Increased baseline levels of S100β and LDH in patients on autologous tumor cell vaccine were predictive of poor DFS and OS. However, only persistent rise in levels of S100β significantly predicted overall survival. Thus, S100β is a much better predictive biomarker of treatment response than its other utilities [69].

S100β may have survival prediction utility in stage IV melanoma patients on temozolomide alone or with immunotherapy. Normal initial S100β levels were associated with higher response rates and fewer metastatic sites and better OS. This prognostic feature was, however, lost in multivariate analysis. Initially, S100β levels rose in almost all patients on systemic therapy; however, those with rapid normalization experienced prolonged survival. Both S100β and LDH levels after treatment were informative in this cohort [70].

1.4.6.2 Serum LDH as Melanoma Biomarker

Lactate dehydrogenase (LDH) is a glycolytic enzyme that catalyzes the conversion of pyruvate to lactate. There are five isoenzymes, namely, LDH1, LDH2, LDH3, LDH4, and LDH5, with specific tissue distributions; LDH 1 (heart), LDH2 (reticuloendothelial cells), LDH3 (lungs and some other tissues), LDH4 (pancreas, kidney, and placenta), and LDH5 (liver and skeletal muscles). Levels of the various isoenzymes can rise as a result of damage to any of these tissues. This has been the basis for the use of elevated serum LDH1 in diagnosis of myocardial infarction. But systemic ischemia can lead to release of LDH from other different sources.

Apparently, elevated serum LDH in stage IV melanoma patients is due to LDH3 and LDH4, in association with decreased LDH1 and LDH2 isoenzymes [71]. However, in metastatic cancers, levels of all isoenzymes can rise. Cells do not secrete LDH; as such, the mechanism of its release into the circulation is uncertain. The hypoxic conditions created as cancer cells outgrow their vascular supply could potentially lead to increased LDH expression to meet their glycolytic phenotype. Under such stressful conditions, dead cells will release LDH into the extracellular environment that eventually enters the circulation via nearby capillaries.

Circulating LDH levels are prognostic and possibly therapy response predictive biomarkers of metastatic malignant melanoma. Because of its strong prognostic relevance, this biomarker is incorporated into the American Joint Committee on Cancer (AJCC) melanoma staging system, as a biomarker that complements definition of the M stage. In metastatic disease, an elevated LDH indicates M1c designation irrespective of metastatic site.

Serum LDH can be elevated if hemolysis occurs prior to sample processing. Hence, false positive test result can occur. To avoid this, it is recommended that two or more samples be taken at least 24 h apart. Samples can be stored at 4 °C for up to 3 days or frozen for 10 days and still give reliable results. Systemic inflammation, ischemia, hepatitis and other infections, immunologic diseases, and other conditions can lead to nonspecific release of LDH into the circulation.

Serum LDH levels have been extensively investigated with regard to melanoma tumor biology. As a tumor biomarker, LDH is elevated in several tumors. Work by Hill and Levi in 1954 first uncovered the activity of LDH to be elevated in sera from patients with neoplastic disease and in pregnant women, but low in healthy individuals and those with other pathologies [72]. Thus, LDH is assayed in sera from patients with other malignancies such as ovarian and breast cancer. However, Finck and coworkers first revealed the prognostic relevance of LDH in malignant melanoma [73]. Their study included patients with stage II and III melanomas who had liver metastasis (reviewed histories and hospital records of these patients). As a predictor of disease recurrence, serum LDH achieved a sensitivity and specificity of 72.1 % and 97.0 %, respectively. A sensitivity of 95.1 % and specificity of 82.8 % were achieved in predicting liver metastasis in stage II disease, and for stage III disease, these values were 86.5 % and 57.1 %, respectively. Patients with elevated LDH levels had a mean survival of only 5.9 months. The authors concluded that "monitoring of serum LDH can provide useful information in the postoperative follow-up of patients with melanoma" [73].

Serum LDH as Melanoma Prognostic and Treatment Response Biomarker
LDH has also received attention as a prognostic and treatment response biomarker in patients with malignant melanoma. In a retrospective study of metastatic melanoma, pretreatment LDH levels were significant independent predictor of survival [74]. Normal LDH in metastatic cancers also predicts good outcome [75].

The predictive value of LDH in two large randomized case–control studies (GM301 and EORTC 18951) of advanced melanoma patients on dacarbazine and/or oblimersen was investigated. In both studies, baseline LDH levels were elevated. Survival was poor in patients with elevated LDH. Elevated LDH was not correlated with tumor size nor associated with any disease site. Importantly, LDH levels were highly predictive of oblimersen effect [76]. Serum LDH as a predictive biomarker of plitidepsin and dacarbazine therapy of advanced stage melanoma revealed that all responders to therapy had normal LDH levels at baseline. Median PFS was longer in those with normal baseline LDH [77].

The prognostic use of serum biomarkers for leptomeningeal spread of malignant melanoma indicated that lower serum LDH (<240 units/L) was associated with overall survival of 7.8 months compared to 3.5 months for those with elevated levels. This remained a significant predictive factor of OS together with Karnofsky Performance Status (KPS) in multivariate analysis [78]. Visceral metastasis (other than lung) (HR 1.8), elevated serum S100β (HR 1.7), and elevated LDH (HR 1.6) were negatively associated with survival in patients with metastatic melanoma. Favorable survival after metastasectomy was more likely in patients with normal serum levels of LDH and S100β (5-year survival was 37.2 %) [79].

1.4.6.3 Serum MIA as Melanoma Biomarker

Melanoma inhibitory activity (*MIA*) gene on chromosome 11q13.2 encodes an 11 kDa extracellular soluble protein. It is a member of four homologous gene family, including *OTOR* (*FDP, MIAL*), *MIA2*, and *TANGO*. Melanoma cells, chondrocytes, chondrosarcomas, and some adenocarcinomas (e.g., breast and colon) express MIA. Melanoma inhibitory activity protein is characterized as an autocrine growth inhibitory factor. It interacts with the extracellular matrix and cell adhesion receptors. In functional studies, increased expression in transfected melanoma cell lines resulted in acquisition of invasive and metastatic phenotypes. It also interacts with integrin α4β1 on leukocytes, interfering with their anticancer immune functions. Various studies suggest serum MIA has utility in clinical staging of melanoma, detection of disease progression from localized to invasive stages, and for monitoring response to therapy in advanced stage melanoma.

Melanoma inhibitory activity protein as a prognostic biomarker in monitoring treatment outcome has been explored. In the study by Meral et al., mean levels of MIA, LDH, and S100β were higher in melanoma patients than controls. MIA levels were much higher in patients with visceral metastasis than in those with only nodal metastasis. A decline in serum MIA levels following systemic treatment, as well as low LDH levels before treatment, was predictive of favorable outcomes [80].

The levels of MIA can predict disease progression. Serum MIA, S100β, LDH, and ESR were elevated in patients with progressive melanoma, but highest predictive sensitivity was achieved with elevated S100β (91 %) and MIA (88 %). However, LDH had the highest specificity (92 %). In multiple logistic regression analysis, only LDH was a statistically significant biomarker of disease progression, with no additional predictive value provided by incorporating S100β or MIA. This observation was because of the strong correlation of these biomarkers to LDH [81]. In another study, patients with positive sentinel lymph nodes had mean MIA levels almost twice (14.53 ng/ml) as high as those from patients without nodal involvement (7.32 ng/ml). Clarke and Breslow classification was unable to differentiate between the two groups, suggesting MIA may be superior to these metrics at detecting sentinel lymph node spread [82].

Melanoma inhibitory activity as a melanoma recurrence biomarker in early stage I/II disease has been explored. As many as 5334 serum samples from 1079 stage I/II patients were analyzed. Melanoma inhibitory activity performance in detecting metastasis was sensitive at 67.6 % for stage I and similar for stage II (65.6 %) disease. Specificities were 76.9 % for stage I and 66.7 % for stage II disease. However, false positive rate was significant in older women and men with high Breslow thickness, cautioning interpretation of data in these individuals [83]. Serum markers for the detection of early recurrence or relapse in stage III melanoma patients revealed that high MIA levels (12.55 ng/ml) were associated with lymph node metastasis. Levels increased with the number of nodal involvement (levels in those with three or more nodes involved were much higher than those with one or two nodes). Recurrence risk was five times higher in stage III

patients with MIA greater than 12 ng/ml. The mean MIA level in the relapse group was 13.76 ng/ml compared to 7.52 ng/ml in the group without recurrence [84].

Serum MIA measurement has demonstrated utility in clinical staging and therapy response monitoring. Increased serum levels were detected in 13 % of stage I, 23 % of stage II, and 100 % of stage III or IV patients. MIA outperformed S100β in delineating these stages. Response to therapy in stage IV patients was associated with changes in serum levels. Importantly, metastasis in early stage disease was associated with increased MIA levels [85]. Increased serum MIA levels correlated with clinical stage. Positive serum levels were detected in 5.6 % of stage I/II, 60.0 % of stage III, and 89.5 % of stage IV patients. The levels decreased with treatment (surgery, radiation, or chemotherapy) and were elevated in those with metastasis [86].

The utility of MIA in monitoring patients on therapy with polyvalent melanoma vaccine, IFN-α2β, and IL-1 is encouraging. At completion of therapy, MIA levels were much higher in patients with progressive disease than in those responsive to treatment. A significant increase was observed overtime with disease progression regardless of the type of treatment. Levels of MIA were elevated sooner than clinical evidence of disease recurrence [87].

In uveal melanoma, metastasis is associated with marked increase in serum MIA. Nonmetastatic patients had values of about 6.6 ng/ml compared to 26.28 ng/ml in those with metastasis. It does appear useful for monitoring metastasis of uveal melanoma [88].

1.4.6.4 Serum VEGF as Melanoma Biomarker

Vascular endothelial growth factor (VEGF) is a potent mitogen for endothelial cells and, in tumors, is an established neoangiogenic growth factor that stimulates growth, proliferation, migration, invasiveness, and metastasis of cancer cells, including melanoma cells. Levels are increased in hypoxic conditions characteristic of growing solid tumors. Because of its role in cancer biology, and the findings that expression of VEGF and angiogenesis are prognostic factors in solid tumors, serum levels have been explored as prognostic and predictive biomarker of melanoma.

A number of angiogenic growth factors, including VEGF, βFGF, IL-8, and angiogenin (ANG), are significantly elevated in blood from melanoma patients compared to healthy controls. VEGF, βFGF, and IL-8 levels are associated with advanced stage disease and tumor burden. Cytostatic treatment caused increased βFGF, IL-8, and ANG (but not VEGF) levels. Univariate analysis showed VEGF, βFGF, and IL-8 could predict poor PFS and OS. In multivariate analysis, these three biomarkers, as well as tumor burden, were independent predictors of OS, but for PFS, tumor burden, VEGF, and IL-8 levels were significant predictors [89]. Osella-Abate and colleagues related serum VEGF levels to CTCs and tumor progression. VEGF levels were significantly higher in melanoma patients, especially those with metastasis (similar to Ugurel et al. [89]). During follow-up of stage I–III patients, increasing VEGF levels were associated with relapse. CTCs correlated with serum

VEGF levels, and the presence of both factors was associated with a relapse rate of 81 %. However, in multivariate analysis, CTCs at baseline (but not VEGF as observed by Ugurel et al. [89]) was predictive of OS and time to progression [90]. Another supporting evidence of the fact that serum VEGF levels are high in melanoma patients was provided by Pelletier et al. [91]. Here, significant differences were observed between stage I–III and stage IV patients. Baseline VEGF levels were not elevated in those who relapsed. Of 35 stage I–III patients whose disease progressed, VEGF levels increased in 20 (57.1 %), and this finding had a specificity of 78 %. Importantly, this finding had a negative predictive value of 90 % and could help identify patients in remission (i.e., absence of elevated VEGF) [91]. High serum VEGF is associated with shorter disease-free survival (DFS) compared to those with lower levels (median DFS of 25 months vs. 60 months) [92]. Serum VEGF and BCL2 were significantly elevated in patients compared to controls. VEGF levels were associated with Breslow thickness and mitotic index but not clinical disease stage [93].

Circulating VEGFC has been linked to melanoma metastasis. Patients with distant site metastasis had significantly higher (2584 pg/ml) VEGFC levels than those with subcutaneous spread (1643 pg/ml, $p = 0.0033$). Levels above the median (1500 pg/ml) were significantly associated with deep lymph node involvement (OR, 3.763). No relation to survival or treatment response (dacarbazine with IFN-α or BOLD with IFN-α) was noted [94]. The VEGFC receptor, VEGFR-3 (or Flt-4), was assayed in sera from patients and correlated with clinicopathologic features. By immunohistochemistry, melanoma cells expressed high levels of the receptor. In agreement, the serum levels were significantly elevated in melanoma patients than controls ($p = 0.00001$). The elevated levels were associated with high tumor burden and in nonresponders, while low circulating levels associated positively with DFS [95].

Serum VEGF has been shown to be a predictor of response to high-dose IL-2 treatment in patients with melanoma and renal cell carcinoma. These investigators used a multiplex antibody-targeted protein array platform to profile biomarkers relevant to tumor immunobiology. In a training set, 68 biomarkers were identified and tested on an independent validation set. Eleven biomarkers were predictive of treatment outcome. In a multivariate permutation test, however, VEGF and fibronectin were independent predictors of response to IL-2 treatment. Lack of response and poor OS was associated with high levels of these biomarkers [96].

1.4.6.5 Serum Levodopa to L-Tyrosine Ratio as Melanoma Biomarker

The search for other circulating biochemical markers for melanoma led to the evaluation of melanin precursors in plasma and serum of patients. Specifically, Levodopa or L-dopa to L-tyrosine (LD/LT) ratio, which is a measure of tyrosinase functional activity, has clinical utility. In a pilot study, the LD/LT ratio was elevated in patients with stage III (15.23×10^{-5}) compared to stage I disease (10.88×10^{-5}). The levels were much higher in those with stage IV (45.7×10^{-5})

disease, which was significantly different from the other stages. Increased ratio was associated with the number of metastatic sites (metastasis causes increase L-dopa in association with decreases in L-tyrosine levels) [97]. In another study, the LD/LT ratio was noted to perform less well compared to S100β in melanoma detection, with a sensitivity of 51 % compared to 66 % for S100β. The ratio was significantly higher in stage IV disease compared to the other stages and increased with disease progression. For prediction of disease progression, LD/LT ratio had a sensitivity and specificity of 78 % and 67 %, respectively, which was comparable to S100β (74 % sensitivity and 83 % specificity) [98]. In a follow-up study, the ratio was assessed for various clinical uses (treatment monitoring, prognosis, and follow-up monitoring for disease progression). Stage III disease patients had decreased levels of S100β after surgical lymph node dissection, but the LD/LT ratio did not decrease. However, treatment with chemotherapy was associated with 38 % reduction in the ratio and 45 % decrease in the levels of S100β. High LD/LT ratio in stage IV disease patients at inclusion in the study conferred shorter survival time (3 months) compared to patients with lower ratios (15 months) [99]. A multi-biomarker assay including LD/LT ratio, LDH, MIA, and S100β was investigated for performance alone or in combinations as panels. S100β and MIA levels were highly correlated, especially in stage IV disease. S100β in combination with the LD/LT ratio had the highest sensitivity of 73 % and specificity of 70 % for detection of stage III/IV disease. Among the biomarkers, only the LD/LT ratio increased with disease progression from stage I–III to stage IV. However, levels of MIA, S100β, and LDH (but not LD/LT ratio) predicted dismal survival of stage IV patients. S100β and MIA were the best survival predictors [100].

1.4.6.6 Serum Cysteinyldopa as Melanoma Biomarker

5-S-cysteinyldopa (5-S-CD) is a precursor of pheomelanin. Circulating 5-S-CD levels are usually within the normal range in early stage melanoma patients, and hence is not an early detection biomarker. However, levels are significantly elevated in circulation of patients with advanced stage disease, and have potential utility in monitoring disease progression and prognostication. The large-scale study by Wakamatsu et al. of serum 5-S-CD in relation to melanoma progression is informative [101]. They collected 2648 serum samples from 218 patients for evaluation. In stage IV disease patients, circulating levels were significantly much higher than the normal upper limit of 10 nmol/L. In this series, its utility for predicting distance metastasis achieved a sensitivity of 73 % and specificity of 98 %, with an equally impressive positive predictive value of 94 %. Of the 1480 samples from patients with nonmetastatic disease, 5 % had elevated 5-S-CD levels suggesting a possible risk of impending metastasis in these patients. Importantly, 33 % of the patients had elevated levels prior to clinical detection of visceral metastasis, while 37 % had coincident elevation with detection of metastasis. As a prognostic predictor, elevated serum 5-S-CD before surgery conferred

significantly reduced survival. Of note, an elevated level in chronic kidney disease patients is not necessarily a measure of melanoma progression.

1.4.6.7 Serum Autoantibodies as Melanoma Biomarkers

Novel technologies have been developed for serum autoantibody detection and capture in patients with melanoma. A proteome serology to complement expression library-based approach has been used for discovery of tumor-associated antigens. This technology, serological identification of antigens by recombinant expression (SEREX), uses the patient's immune response for biomarker identification. Sera from 94 patients were screened for anti-melanoma reactivity, of which seropositivity was detected in 66 % of the patients (2–6 antigens by 1D and average of 2.3 per case for 2D Western blot approach). Spot identification by MS resulted in 18 antigens, of which 17 were new. These proteins include galectin 3 (known to be involved in cancer invasiveness and metastasis) and enolase, which is deregulated in cancer [102]. To identify early diagnostic biomarkers, this group used a modified serological proteome approach (SERPA) to screen sera from patients. Briefly, protein extracts from G361 melanoma cell line were separated by 2D gel electrophoresis, membrane blotted and incubated with sera from melanoma patients. Positive reactive spots were subjected to TOF MS analysis. From a total of 13 positive spots, five proteins, eukaryotic elongation factor-2 (EEF2), enolase 1 (ENO1), aldolase A (ALDOA), GAPDH, and heterogeneous nuclear ribonucleoprotein (HNRNP) A2B1, were uncovered. Additional evidence of their tumor association was the finding that G361 and other melanoma cell lines expressed the mRNA of three of these proteins [103].

A natural glycoprotein microarray was developed for serum autoantibody profiling. Dual lectin affinity chromatography was employed to capture glycoproteins in melanoma cell lines. Separated proteins were spotted on nitrocellulose slides and reacted with sera from melanoma patients. Nine fractions were very specific (100 %) at differentiating lymph node-positive from lymph node-negative patients at a modest sensitivity of 55 %. These fractions included GRP94, ASAH1, CTSD, and LDHB [104]. In a follow-up study, this microarray coupled with mass spectrometry enabled the confirmation of the five melanoma-associated antigens (GRP75, GRP94, ASAH1, CTSD, and LDHB) initially discovered. Their predictive value indicated GRP94 was significantly positively, while LDHB, ASAH1, and CTSD were negatively associated with nodal status. Multivariate analysis indicated the presence of anti-GRP94 and absence of anti-LDHB, anti-ASAH1, and anti-CSTD, as well as Breslow thickness could predict nodal status. These proteins have potential for stratifying clinically node-negative patients prior to sentinel lymph node biopsy [105].

Other emerging serum biomarkers of potential interest in clinical management of melanoma include CRP, MMP (1 and 9), cytokines (IL-6, 10, sHLA), integrins, ICAM1, CD44, other melanoma-associated antigens, albumin, pyruvate kinase type M2, and TA-90 immune complex.

1.4.6.8 Circulating Melanoma Proteomic Biomarkers

Proteomic technologies have been used to identify melanoma protein and peptide signatures in circulation. Proteomic approach enabled the identification of autoantibodies to α-enolase and γ-enolase in sera from melanoma patients treated with alkylating agents and IFN-β [106]. MALDI-TOF MS was used to uncover transthyretin, angiotensinogen, and vitamin D-binding protein as melanoma biomarkers. Transthyretin and angiotensinogen were upregulated, while vitamin D-binding protein was downregulated in melanoma patient samples. One month after surgical removal of stage I and II tumors, these protein levels returned to normal, indicating their association with the disease [107].

Melanoma diagnostic biomarkers have been identified by proteomic technologies coupled with artificial neural network. Biomarker signature enabled differentiation of stage IV patients from controls at specificities of 92 % (for protein ions) and 100 % (for peptides). Ninety eight percent (98 %) of stage I disease patients were discriminated from stage IV disease. Sequencing revealed the peptides to originate from metastatic-related proteins including alpha 1-acid glycoprotein precursor-1 and alpha 1-acid glycoprotein precursor-2 (AAG-1 and AAG-2) and complement C3 component precursor-1 (CCCP-1). Immunoassay confirmed significant elevation of AAG-1 and AAG-2 in sera from stage IV disease patients compared to controls [108]. To identify biomarkers for early stage disease, and predictive of tumor behavior, sera from stage I and IV patients were subjected to MALDI-TOF MS. Additional samples from stage III patients with regional lymph node surgery, who were stable or relapsed during a 1 year of follow-up, were examined. Correct stage classification was achieved in 88 % of the patients. Eighty percent (80 %) of stage III disease patients were correctly predicted as progressing or not. Importantly, 82 % of stage III patients with progressive disease could be predicted by this proteomic approach, compared to only 21 % identified by S100β [109]. Another study for identification of early detection serum biomarkers by proteomic profiling is noteworthy. Samples from controls and all stages of melanoma were subjected to SELDI-TOF MS. The informative proteins enabled good classification between cancer and controls, as well as stage stratification. In a validation cohort, the diagnostic accuracy was 98.1 % (96.7 % and 100 % for sensitivity and specificity, respectively). Early stage disease (stage I/II) was correctly classified at 100 % accuracy [110].

MALDI-TOF MS serum profiling for prognostic biomarkers of melanoma has also been explored. In the Findeisen et al. study, sera from stage I and stage IV disease patients were first profiled, which enabled identification of serum amyloid A (SAA) as a candidate biomarker. This was then assayed in patient sera using immunoassay and compared to established biomarkers including S100β, LDH, and CRP. Serum amyloid A levels correlated with poor survival, being a significant prognostic factor for stages I–II ($p = 0.043$) and stage IV ($p = 0.000083$) disease patients. The prognostic performance was enhanced for stage I–III diseases by incorporating CRP levels ($p = 0.011$). Multivariate analysis identified SAA,

CRP, S100β as well as sex, stage, and tumor load as independent prognostic factors. For stage I–III disease, the panel of SAA and CRP performed better than S100β as a predictor of DFS and OS [111]. SELDI-TOF MS for identification of recurrence biomarkers in serum from early stage disease (I/II) patients detected protein peaks between 3.3 and 30 kDa that could differentiate patients with recurrences from those without. Expression pattern differences of three proteins were used to create a classification tree. This protein signature had a sensitivity of 72 % and a specificity of 75 % in predicting disease recurrence [112].

Hydrogel core shell nanoparticle technology was used to selectively capture, enrich, and protect low-abundant serum proteins. This initial proof of principle study enabled the identification of BAK as a differentially expressed biomarker between patients with melanoma and those with nevi. Increased serum BAK levels were associated with lesions having junctional activity, and weak BAK expression was associated with "sparse" dermal nests detected by confocal microscopy [113].

1.4.7 Circulating Melanoma Cells

Melanoma tends to be aggressive with early lymphatic and hematogenous spread. The single most important factor in cancer mortality is distant metastasis with evolution of clones resistant to initially effective therapy. While generally known to be aggressive with high propensity to metastasize, the clinical course of melanoma can be unpredictable. Primary tumor thickness is the most important prognostic factor; however, ulceration, locoregional nodal involvement, and distant metastasis are also prognostic variables. Although these are helpful predictive factors, clinical course can still remain illusive. The detection of metastasis for clinical staging requires biopsy and comprehensive scanning of patients. Circulating melanoma cells (CMCs) or "liquid biopsy" offers attractive sample source that can be obtained serially even in patients who are not suitable candidates for surgery to obtain tissue biopsy. Circulating melanoma cells enable detection of micrometastatic disease. Additionally, CMCs can help with prognostication, treatment prediction and monitoring for treatment effect, disease progression, recurrence or even evolution of new clones, and tumor biology. Circulating melanoma cell enrichment, isolation, and/or detection have relied mostly on use of molecular and cytometric techniques (protein- or size-based assays).

1.4.7.1 Molecular Approaches to CMCs

The PCR method has been extensively employed for detection of CMCs. This involves targeting the expression of putative melanoma-specific transcripts that serve as surrogates of CMCs. The rational for this approach is that the chosen targets are specific to the melanocyte lineage, indicating their absence in tissues without melanocytes. For example, the presence of tyrosinase mRNA (restricted to

the melanocyte lineage) in lymph nodes is indicative of disseminated melanoma cells. Moreover, mRNA is supposedly labile in circulation; hence, their detection is more likely from enclosed (CMC) sources. The caveats to these assumptions are that mRNA can also be protected in exosomes and other microparticles and hence may not necessarily be indicative of CMCs. Other limitations of PCR-based CMC detection include:

- There could be high false negative rates. Positive detection relies on the number of CMCs expressing the targeted surrogate marker. Tumor heterogeneity indicates not all cells of the primary melanoma will express the marker. Also clonal evolution with metastasis could lead to emergence of clones not expressing the marker used for detection.
- CMCs cannot be enumerated for predictive clinical use, but quantification, which may mirror target copy numbers and possible number of cells, may overcome this limitation.
- Leukocyte nucleic acids could dilute CMC targets, which could result in false negative results.
- There is lack of analytical standardization leading to inconsistent results even when the same target is being assayed.
- Issues endemic with PCR including primer and molecular probes variability, interlaboratory protocol differences, sample handling, different efficiencies of reverse transcriptase enzymes, processed pseudogene interference, and illegitimate transcription are all problematic.

Nevertheless, PCR has its strengths compared to cytometric-based methods. The technique can be sensitive and specific in detection of CMCs. It is estimated at being able to detect one malignant cell per 10^6–10^8 normal white blood cells or one malignant cell per 1–10 ml of blood [114]. Given the rarity of CTCs (1 per 10^6–10^7 normal WBCs), this may have superiority over cytometric detection of CTCs. With good primer design, PCR can be very accurate with reference to specific detection of intended target compared to possible cross-reactivity of antibodies used in cytometric immunoassays. Thus, some progress has been made using PCR to target transcripts of markers such as tyrosinase, MART-1/MELAN-A, and MITF-M isoform.

Tyrosinase mRNA as a Marker of CMCs

The first report in 1991 of CMCs by Smith et al. targeted tyrosinase (*TYR*) mRNA using nested RT-PCR [115]. Indeed, melanoma was the first solid tumor where RT-PCR was used to indicate the presence of CTCs. Tyrosinase is the first enzyme in the melanin production pathway and hence expressed by pigment-producing cells. Low levels are expressed by normal colonic and testis tissues, and by colon and brain cancer cells.

While some initial reports had technical and methodological issues, many assays indicate its clinical usefulness alone or in panels for CMC detection in advanced

stage melanoma. Detection has been associated with prognostic variables such as disease recurrences and overall survival. The work by Quaglino et al. indicated that the presence of tyrosinase mRNA in two consecutive samples measured over a 3-month period was indicative of visceral invasion [116]. But meta-analysis of several works (from 1996 to 1999) indicated only 45 % of all AJCC stage IV patients were tyrosinase positive, making its use less generalizable and unattractive for monitoring hematogenous spread and prognostication [117].

MART-1 mRNA as a Marker of CMCs

Melanoma antigen recognized by T cells 1 (MART-1) also known as MELAN-A antibody (MLANA/MELAN-A) is involved in melanin biosynthesis and has been used as a lineage marker of melanocytes. Its usefulness in differential diagnosis of melanoma is well documented. MART-1 has most frequently been studied in conjunction with tyrosinase, as a panel to improve detection of CMCs. Detection is infrequent in early stage disease. In one study of stage I/II melanoma patients, circulating *MART-1* and *TYR* transcripts were independent prognostic factors of DFS in only those with disseminated or locoregional relapse [118]. *MART-1* is associated more with advanced stage disease, and circulating levels correlate with tumor size and stage. In 1999, Palmieri et al. detected CMCs as biomarker of tumor progression. A later study, including *MART-1* and *TYR*, in a panel offered no additional predictive value to established clinical prognostic parameters [119]. *Tyrosinase*, *p97/VCP*, or *MELAN-A* positivity of serial samples indicated that patients with prolonged CMCs had evidence of disease progression or harbored more aggressive disease [120].

MITF-M mRNA as a Marker of CMCs

MITF is involved in melanocyte proliferation and differentiation and also controls melanoma cell proliferation and invasion. The melanoma-specific isoform (MITF-M) is the target for CMC detection. Circulating levels of *MITF-M* transcripts are significantly correlated with disease progression and OS. In addition to its possible role in detection of micrometastasis, circulating *MITF-M* is predictive of treatment outcome [121]. In combination with tyrosinase, CMCs were detected in stage III and IV disease patients at a frequency of 25 % and 38 %, respectively [122]. Circulating *MITF-M* levels predicted disease stage and progression. Low levels were associated with invasive but non-proliferating CMCs [123]. But signals that enhance MITF-M activity can convert these cells into highly proliferating CMCs. Because *MITF-M* expression targets and induces the expression of some melanoma target genes including *TYRP1* and *PMEL17/SP100*, the use of these genes to detect CMCs requires *MITF-M* expression.

Other markers of melanogenesis are used in CMC detection. TYRP1 and TYRP2/DCT are involved in melanin synthesis. In advanced stage melanoma,

89 % of blood samples were positive for *DCT*, and this was associated with tumor thickness and patient outcome [124]. Additionally, 25 % of sentinel lymph nodes could be upstaged with *DCT* analysis [125].

1.4.7.2 CMCs in Advanced Stage III and IV Melanoma

The RT-PCR techniques targeting single or multiple melanoma expressing transcripts have been used to evaluate the prognostic value of CMCs in advanced stage disease. The detection rates have been variable, due to multiple factors. In general, rates tend to be higher with the use of multiple markers compared to single markers, and with serial sampling compared to single baseline sampling. Reported rates have ranged from 14 to 80 %. In spite of these issues, the prognostic value of CMCs in stage III and IV melanoma has been encouraging.

Multiple studies of stage I–III diseases concur that CMC presence is associated with early relapse, shorter DFS, and OS. Three prospective clinical trials evaluated the clinical utility of CMCs in regard to prediction of recurrence and treatment response [126–128]. In the study by Scoggins et al., 820 patients were included. Patients had sentinel lymph node biopsy before lymphadenectomy, and samples were collected serially beginning at the time of sentinel lymph node biopsy. Twenty five percent (207 patients) had stage III disease. RT-PCR targeting *TYR*, MELAN-A, MAGE3, and *GP-100* was positive in 14 % of the stage III patients (115 patients). Patients who were positive for >1 marker during the serial sampling had shorter DFS and OS compared to those with only one positive marker. A nested study of patients enrolled in the EORTC 18991 phase III trial included serial sampling of patients for CMC evaluation. The trial compared patients on pegylated interferon-alpha2b with observation. CMCs defined as tyrosinase or *MELAN-A*-positivity were detected at a rate of 36.5 % (109/299). Cox time-dependent analysis of prognostic factors revealed that detection of single positive CMCs at any time conferred significantly high risk of developing distant metastasis (HR $= 2.25$, $p < 0.001$). Hashimoto et al. assessed CMCs in stage III patients who had complete lymphadenectomy prior to adjuvant vaccine treatment. Samples taken only once after surgery were subjected to multi-marker RT-PCR. While CMC presence did not correlate with prognostic factors, in multivariate analysis, patients with ≥ 2 positive markers had significantly worse DFS (HR $= 2.13$; $p = 0.009$).

In stage IV disease, a number of studies reveal similar prognostic significance of CMCs [116, 129, 130]. The study by Quaglino et al. included 149 patients on medical treatment (chemotherapy or chemotherapy with immunotherapy) and 51 post-metastasectomy patients [131]. Samples were collected before intervention and serially thereafter for single marker (tyrosinase) RT-PCR. Overall, all surgical cases were negative for CMCs at baseline, and 45.1 % were positive on serial analysis. CMC dynamics were clinically informative. Patients who converted from CMCs positive to negative demonstrated response, whereas metastasis was associated with CMC positivity (negative conversion to positive or positive throughout). Multivariate analysis revealed that CMCs at baseline and during follow-up were

significantly associated with adverse time to progression and OS. Patients with baseline CMCs had time to progression HR of 1.45 ($p = 0.046$) and OS HR of 1.57 ($p = 0.024$). Also a follow-up CMC-positivity was associated with a time to progression HR of 3.60 ($p < 0.001$) and OS HR of 4.83 ($p < 0.001$). In the MMAIT-IV study, whereby patients received metastasectomy before randomization into vaccine therapy or placebo, RT-PCR targeting *MAGEA3*, *MART-1*, and *PAX3* transcripts in 244 patients was used to evaluate CMCs. Samples were collected before surgery and at 1 and 3 months post. CMC detection rate was 54.1 % at baseline, and in multivariate analysis, the presence of ≥1 positive marker was associated with worse DFS (HR = 1.64, $p = 0.002$) and OS (HR = 1.53, $p = 0.028$). In the subset of patients with multiple samples (214 patients), again multivariate analysis revealed that CMC detection at any time point conferred worse DFS (HR = 1.91, $p = 0.020$) and OS (HR = 1.91, $p = 0.012$).

The CELLSEARCH® system was used to evaluate CMCs (anti-CD146) in 101 patients with metastatic or inoperable stage IV disease (78.2 % were stage M1c) [129]. CMCs enumerated ranged from 0 to 36 (mean of 2). In both univariate and multivariate analysis, CMC-positivity (≥1 CMCs) was associated with poor prognosis. With a defined cutoff of two CMCs, patients with <2 CMCs had significantly better OS than those with ≥2 CMCs (7.2 vs. 2.6 months, HR = 0.43, $p = 0.009$). A subset of the patients on treatment (45 patients) had serial CMC enumeration, and this could predict therapy response. Increasing CMC cell count was associated with disease progression, while decreasing counts predicted therapy response. Overall survival was worse in patients with >2 CMCs at any time during treatment.

1.4.7.3 CMCs in Uveal Melanoma

While neural crest cells give rise to both cutaneous and ocular melanoma, the two diseases exhibit different biologic behaviors. For instance, cutaneous melanoma often metastasizes via lymphatic vessels to regional lymph nodes. However, due to the absence of lymphatic vessels in the uveal tract, ocular melanoma spreads via the blood stream, thus making CMC detection attractive. Additionally, uveal melanoma is more likely to spread to the liver and lungs and less likely to harbor *BRAF* and *CDKN2A* mutations than cutaneous melanoma. However, both express similar markers, and hence, identical methodologies have been used to study CMC in both diseases.

A number of studies have evaluated the prognostic utility of CMCs in ocular melanoma, and almost all support a prognostic role for their presence. In a prospective longitudinal study, CMC detection rate targeting *TYR* and *MELAN-A* mRNA in uveal melanoma increased with multiple sampling, leading to the detection in almost all patients (96.7 %). While prognostic relevance was not demonstrated, this observation is encouraging as it suggests serial sampling can afford valuable clinical decision-making in almost all patients [132]. Boldin et al. had earlier evaluated the prognostic value of CMCs in uveal melanoma [133]. In a

nonmetastatic setting, RT-PCR targeting *TYR* transcripts was detected in 41 patients. Samples were collected before and after treatment. Surprisingly, the majority (69 %) of samples that were positive prior to treatment converted to negative after therapy. CMC-positivity was significantly associated with poor 5-year survival ($p = 0.023$). Schuster et al. evaluated CMCs in patients with metastatic uveal melanoma, also targeting *TYR* and *MELAN-A* mRNA [134]. The presence of CMCs was an independent prognostic factor, conferring worse DFS (HR = 2.2) and OS (HR = 4.0). A dual methodology, using RT-PCR of *TYR* mRNA and ISET technique, was used to assess CMCs and data related to prognostic variables. It was concluded that *TYR* transcript levels correlate with the number of CMCs isolated by ISET method, as well as DFS ($p < 0.05$) and OS ($p < 0.05$). In a larger study by Schuster et al., samples were taken from 110 patients with primary uveal melanoma at time of treatment and during follow-up [135]. In multivariate analysis, the presence of ≥1 positive CMC was significantly associated with increased risk of developing distant metastasis (HR=7.3), and this also conferred >22 times the risk of death from melanoma. Additionally, the presence of at least one CMC was associated adversely with time-to-progression ($p < 0.001$) and disease-specific survival ($p < 0.001$). Mazzini et al. also used the ISET method to evaluate CMCs in 31 patients with nonmetastatic uveal melanoma [136]. Shorter DFS ($p = 0.012$) and OS ($p = 0.017$) were associated with patients who had >10 CMCs/10 ml of blood. Clinicopathologic prognostic features such as tumor-node metastasis, tumor basal diameter, and tumor height also correlated with CMC counts of ≥10/10 ml of blood. Two studies used immunomagnetic enrichment methods to isolate CMCs based on the expression of melanoma-associated chondroitin sulfate proteoglycan (MCSP). While CMC positivity was associated with poor prognostic variables, it failed to predict disease outcomes [137, 138].

1.4.8 Melanoma Extracellular Vesicles

Similar to all exosomes, melanoma-derived exosomes contain biomolecules useful in disease management. Additionally, they have important biologic implications in melanoma progression and metastasis. Melanoma-derived exosomes provide proangiogenic signals that can remodel tissues at sites of eventual metastasis and specifically condition sentinel lymph nodes for subsequent metastasis. Exosomes at lymph nodes produce signals that recruit other melanoma cells. Moreover, they are involved in educating bone marrow progenitor cells to incite tumor growth and metastasis.

The biomolecular composition of melanoma exosomes is identical to all exosomes. These include nucleic acids (importantly miRNA), proteins, lipids, and other metabolites, and their levels are of diagnostic and prognostic relevance. For example, the levels of exosomal MIA and S100β were significantly higher in patients than controls, and correlated with serum levels [139]. As diagnostic biomarkers, exosomal MIA and S100β achieved AUROCC of 0.883 and 0.840,

respectively, and levels of MIA above 2.5 μg/l were an adverse prognostic factor of survival.

1.4.9 Summary

- Melanoma is characterized by a well-charted molecular pathology, which should translate into the development of noninvasive products for disease detection and management.
- The findings of tumor DNA methylation, mutations, and LOH in ctDNA enable safe, comfortable, and effective disease management.
- Serum biomarkers continue to provide useful information in the clinical management of melanoma patients.
- Proteomic efforts and novel technologies for CMC analyses are adding to the number of melanoma prognostic biomarkers.
- Knowledge on tumor biology is important for designing efficient and efficacious therapies. Insights into melanomagenesis and disease progression provided by the study of circulating extracellular vesicles and miRNA are invaluable in this regard.

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Chapter 2
Head and Neck Cancer Biomarkers in Circulation

Key Topics

- Molecular pathology of head and neck cancer (HNC)
- Circulating cell-free nucleic acids as HNC biomarkers
- Circulating HNC miRNA biomarkers
- Circulating HNC cells
- Circulating nasopharyngeal carcinoma biomarkers

Key Points

- The majority of HNCs are squamous cell carcinomas (HNSCCs) and nasopharyngeal carcinomas (NPCs). The etiologic agents of HNSCC are toxins (tobacco and alcohol) and infectious agents from human papillomavirus (HPV), while those of NPC are mostly Epstein–Barr viral (EBV) infections. HNCs have distinct geographic distribution and molecular pathology.
- Circulating biomarkers, especially HNC cells, are potentially valuable in HNSCC staging, prognosis, and prediction of treatment response. While the incidence of HPV-positive HNSCC is on the rise, there is no established diagnostic biomarker for this subtype. There is a need to develop noninvasive biomarkers for accurate detection and management of HPV-positive HNSCC.
- Because of the established etiology, there are numerous biomarkers for NPCs. Validated and in clinical practice are serologic and molecular assays targeting EBV biomolecules. Other potentially useful circulating biomarkers of NPCs are the numerous EBV-encoded miRNAs in circulating exosomes.

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2.1 Introduction

Head and neck cancer (HNC) is the sixth most common cancer with annual global incidence and mortality being 600,000 and 300,000 cases, respectively. The 2016 estimated incidence and mortality cases for the US are 48,330 and 9,570, respectively. They are more common in males than females, with a male to female ratio of about 3:1. High incidence rate of up to 20 per 100,000 is observed in Central and Eastern Europe, Germany, Denmark, Scotland, Italy, Spain, France, Brazil, Hong Kong, the Indian subcontinent, South Africa, and Australia.

Head and neck cancer comprises an anatomic conglomerate of malignancies that arise from the epithelial lining of the upper aerodigestive tract (excluding the thyroid and parathyroid glands). Collectively, tumors of the oral cavity, nasopharynx, oropharynx, hypopharynx, larynx, nasal cavities, paranasal sinuses, and salivary glands constitute HNC. However, the commonest sites are the oral cavity, where many cases (~250,000) are diagnosed annually, and the oropharynx. It should be noted, however, that the etiologic and molecular features differ considerably among these cancer subtypes. For example, salivary gland tumors are biologically distinct. The majority of HNCs (>90 %) is of squamous cell histology, and hence, many studies refer to them as head and neck squamous cell carcinomas (HNSCCs).

Environmental factors and lifestyle exposures such as tobacco and excess alcohol use are known risk factors of oral squamous cell carcinoma (OSCC). Tobacco use is associated with oral and laryngeal cancers, while alcohol use causes more pharyngeal and laryngeal tumors. Additionally, HPV infection of the oropharynx is an etiologic agent for oropharyngeal squamous cell carcinoma (OPSCC), while nasopharyngeal EBV infections account for the majority of nasopharyngeal cancers (NPC), especially in endemic areas. With increasing education and adherence to cessation of tobacco use, the incidence of non-HPV cancers is declining; however, HPV-mediated OPSCC is on the rise, especially in the Western world.

Modern salvage or organ-sparing surgeries coupled with chemoradiation are improving the quality of life of patients with HNC, although no change in the overall survival rate is accomplished due probably to the concept of field cancerization (i.e., locoregional recurrences are common). Additionally, the 5-year survival rate is ~60 %, and this declines with advancing tumor stage. Thus, biomarkers that can be used for early detection (when treatment including administration of chemopreventive remedies is optimal), prognosis, and therapy selection can improve patient management and hence improve survival rates. Noninvasive screening assays targeting biomarkers in saliva or blood in high-risk groups such as smokers, excessive alcohol users, and those involved in oral sex as well as those in endemic areas of EBV infections could lead to early detection, with possible curative interventions, given that only 33 % of all cases are currently detected at an early stage.

2.2 Screening Recommendations for HNC

There are currently no recommended screening guidelines for HNC, as has been established for breast, colon, and prostate cancers. This situation is partly because of lack of evidence that any screening method increases survival rates. There are also no validated blood- or saliva-based tests to detect early HNCs. There is thus the need to develop validated and cost-effective noninvasive tests for HNC.

Individual health centers have their own protocols as to how to screen the population for early cancer detection. For example, the Memorial Sloan Kettering Cancer Center offers free annual head and neck screening to community members. The authorities here also recommend that patients receive annual head and neck examination from their primary care physicians and dentists. This screening should include at least head and neck examination and inspection of the oral cavity and oropharynx. For high-risk individuals, especially those cured of HNSCC, the National Comprehensive Cancer Network has follow-up guidelines to monitor for possible recurrence or development of second primary tumors. These recommendations are periodic physical examination at the following defined frequencies: every 1–3 months for the first year, every 2–4 months for the second year, every 4–6 months during the third to fifth years, and every 6–12 months thereafter.

2.3 The Need for Noninvasive Screening Tests for HNC

The pathology of premalignant or precursor lesions of HNC is well studied. Mostly, OSCC originates from precursor lesions. Oral leukoplakia, which is a white mucosal lesion in the oral cavity (homogenous), and its variant, erythroleukoplakia that is similar to white lesions mixed with red plaques (nonhomogenous), are premalignant lesions of OSCC. These lesions are associated with risk factors such as tobacco and areca nut use. The prevalence of these lesions varies from 0.1 to 0.5 %, and the rate of progression to oral cancer is ~1–2 % per year. Multiple factors including female gender, size of lesion, anatomic location, and presence of dysplasia or erythroleukoplakia determine lesion progression to malignancy. Additionally, the presence of cancer-associated genetic alterations predicts progression. The risk of these lesions developing into cancer includes mutations in *TP53*, loss of chromosome 9p, and decreased cytokeratin 4 and cornulin expression, among other factors. Chromosomal loses at 9p (*CDKN2A* and *PTPRD* loci), 3p (*FHIT* and *RASSF1A* loci), and 17p (*TP53* locus) increase the risk of progression. Molecular assays based on salivary washes or buccal swabs for examination of these genetic changes are commendable and will be easily accepted. Such screening assays are also useful because chemoprevention can be offered to delay or reverse disease progression if detected early.

As the original cancer studied by Slaughter and other workers, the molecular pathway or basis of field cancerization in HNSCC is fairly well characterized.

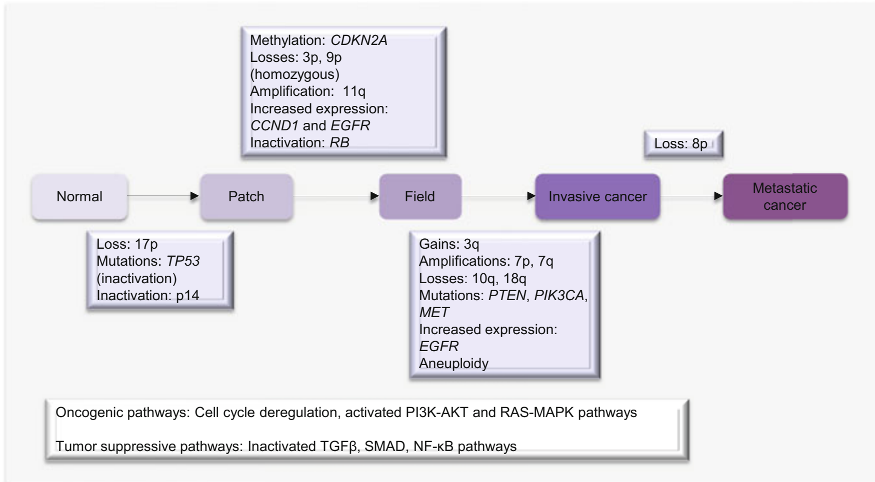

Fig. 2.1 Molecular pathology of field cancerization in HNC

The patch-field squamous cell carcinoma pathway of field cancerization has also been well documented. Mutations or other genetic changes in mucosal epithelium create multiple clonal patches. Gain in growth advantage or evasion of growth control, senescence, and apoptosis leads to expansion of patches. Additional mutations or other genetic and epigenetic events in subclones from these clonal patches can lead to the development of invasive cancer. The genetic sequence of events includes (Fig. 2.1):

- An early loss of chromosome 17p or mutations in *TP53* characterizes clonal patches.
- The pathway to invasive cancer is further driven by homozygous loss of chromosome 9p, 18q, amplification of 11q, mutations in *CDKN2A*, and increased expression of *CCND1*.
- Further amplifications of chromosomes 7p, 7q (high-level amplification), and 3q in association with loss of 10q, activate the EGFR, RAS, and/or PI3K/AKT signaling pathways to drive full malignant transformation.

Because HNSCC has well-established premalignant and precursor lesions, and molecular pathologic progression model, the ability to use biomarkers to detect this cancer early should be easy. A noninvasive test to detect the early precursor lesions targeting molecular genetic alterations should enable early detection and deployment of possible curative interventions. Works at developing such tests using saliva or blood are being explored.

2.4 Molecular Pathology of HNC

Head and neck cancers can originate in any tissue of the head and neck region. However, excluding salivary gland tumors, almost all HNSCCs are from three anatomic regions, the oral cavity, larynx, and the pharynx (Fig. 2.2). Not only is this important for disease management, but this also raises the possibility of early noninvasive detection of many HNCs using oral fluids or blood.

2.4.1 Molecular Classification of HNC

Head and neck cancer has two major etiologic factors, toxins (alcohol and tobacco toxicity) and viral infections. Epstein–Barr viral infection is a risk factor for nasopharyngeal (NPC), while HPV infection causes a subset of HNSCCs. The HPV-negative HNSCCs are associated with tobacco and alcohol use. These subclasses have distinct molecular genetic changes, clinical course, and prognosis. For example, HPV-positive tumors harbor wild-type *TP53* and have favorable outcomes. It is conceivable that the HPV-negative tumors will be genetically

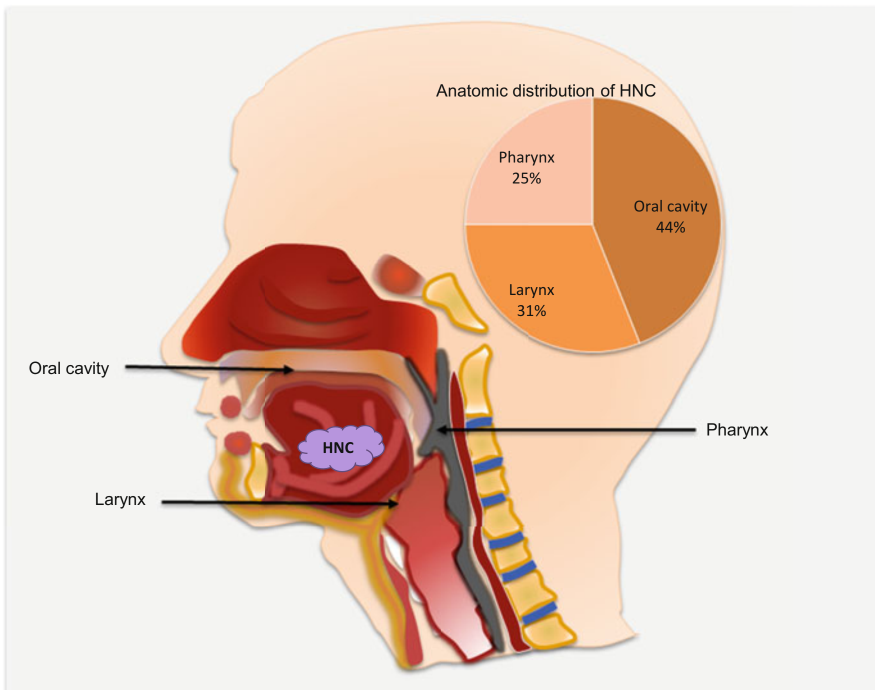

Fig. 2.2 Frequencies and common anatomic locations of HNC

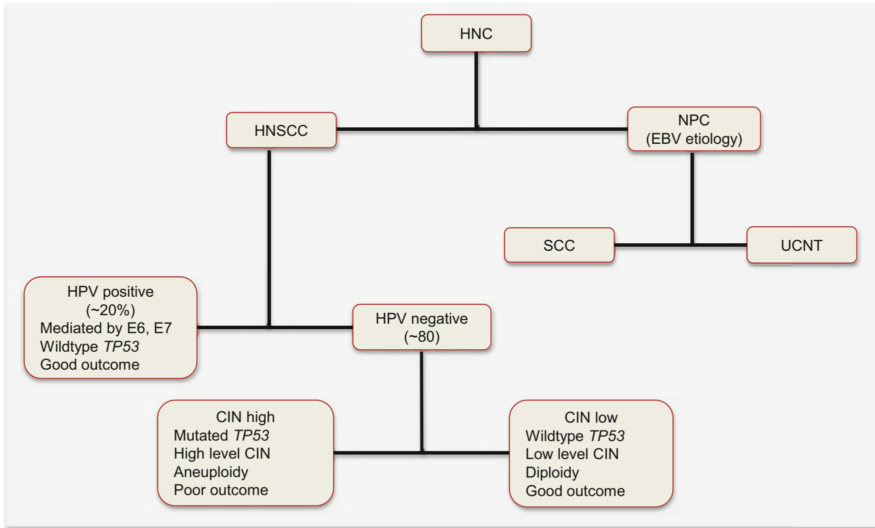

Fig. 2.3 Etiologic classification of HNC with some associated molecular alterations and prognosis. *HNSCC* head and neck squamous cell carcinoma, *NPC* nasopharyngeal carcinoma, *EBV* Epstein–Barr virus, *SCC* squamous cell carcinoma, *UCNT* undifferentiated carcinomas of the nasopharyngeal type

homogenous. However, work by Smeets and colleagues suggests otherwise [1]. Using array CGH, they uncovered three genetically distinct groups of HPV-negative tumors with prognostic relevance. Tumors in "group 1" had hardly any chromosomal instability (CIN) and were associated with wild-type *TP53*, female gender, and nonalcoholics. An intermediate group (group 2) harbored high chromosomal aberrations, while at the extreme end were those with very high levels of CIN (group 3). Patients in "group 1" had the best prognosis, with the worse being among "group 3" patients. On the basis of this and other data, Leemans and colleagues dichotomized HPV-negative tumors into those with high and low CIN (Fig. 2.3) [2]. Tumors with low CIN, which are in the minority (15 %), harbor wild-type *TP53*, are near diploid, and hence are associated with good outcomes. Those with high CIN are mostly aneuploid with *TP53* mutations and are associated with poor prognosis.

An earlier study by Chung et al. using gene expression signatures uncovered four distinct molecular subtypes of HNSCC, also exhibiting different prognosis [3]. These molecular subtypes included tumors enriched for EGFR pathway, mesenchymal cell, normal epithelial-like cell, and high antioxidant enzyme gene signatures. The worse prognosis was among tumors with EGFR pathway gene expression signature.

2.4.2 *Molecular Pathology of HPV-Positive HNC*

High-risk HPV (types 16 and 18) infections of the oropharynx account for ~20 % of all HNSCCs. These cancers are distinct from non-HPV HNSCC, especially at the molecular level. They are associated with wild-type *TP53* genotype, have a favorable outcome, and are mostly confined to the oropharynx. Indeed over 50 % of all oropharyngeal squamous cell carcinomas are HPV positive. In addition to the upper aerodigestive tract, oncogenic HPV also accounts for the majority of cervical cancers, and this epithelial cellular transformation is mostly mediated by viral oncoproteins E5, E6, and E7. These proteins primarily target and deregulate members of the cell cycle and other oncogenic signaling pathways (Fig. 2.4). For example, E5 oncoprotein can interfere with the internalization and destruction of EGFR leading to increased pathway activity. Also the ubiquitin ligase, E6 oncoprotein, targets and degrades p53 tumor suppressor protein to prevent apoptosis, while E7 binds to and inhibits the activity of RB in sequestering E2F to promote cycle progression. Disruption of p53 and RB functions will cause cell cycle progression without mitogen activation. E7 can also promote cell cycle activity by inhibiting CDK inhibitors including p21 and p27.

2.4.3 *Specific Genetic Alterations in HNC*

Head and neck cancer is genetically characterized by abnormalities at almost all chromosomal regions, many of which harbor critical oncogenes and tumor

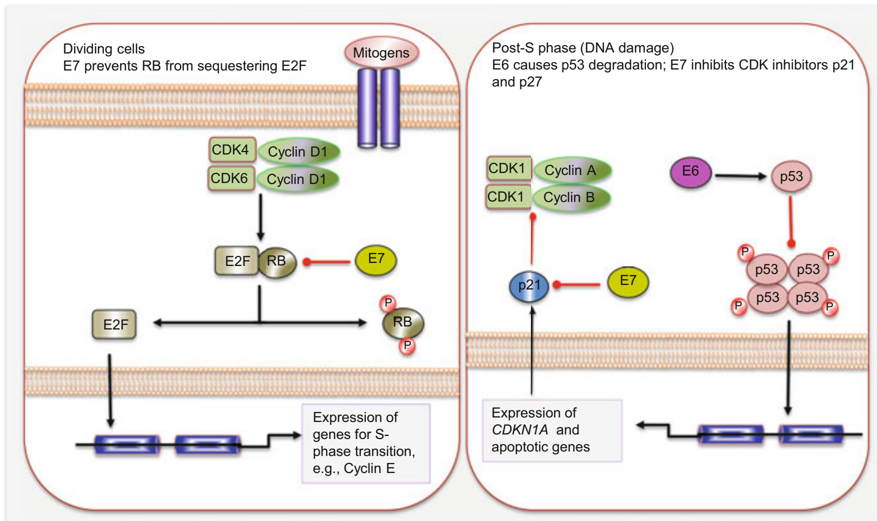

Fig. 2.4 Cell cycle deregulation by HPV E6 and E7 oncoproteins. Red lines with blunt ends indicate inhibitory pathways

suppressor genes. Indeed, the only chromosomes not involved in head and neck carcinogenesis are 12 and 16. The chromosomal changes include amplifications and gains at oncogenic loci, 3q26 (*PIK3CA*), 7p11.2 (*EGFR*), 11q13 (*CCND1*), and 7q31 (*MET*), as well as losses or homozygous deletions of tumor suppressor gene loci including 9p21 (*CDKN2A*), 18q21 (*SMAD4*), 10q23 (*PTEN*), and 17p13 (*TP53*). Together with mutations and epigenetic alterations, the functions of several genes are altered, which eventually reflect on the activities of several carcinogenic signaling pathways including the PI3K/AKT, RAS-MAPK, EGFR, TGFR, p53/RB, and possibly NF-κB pathways.

2.4.3.1 *EGFR* Alterations in HNC

Receptor tyrosine kinase (RTK) signaling responds to growth factor signals to trigger and intergrade the RAS-MAPK and PI3K/AKT pathways that control tumor growth and survival. One of the well-studied RTKs is the ERBB family. The four family members can undergo ligand-mediated homodimerization or heterodimerization to activate the EGFR pathway. Downstream signaling cascade includes the PI3K, RAS-MAPK, phospholipase C (PLC), and JAK/STAT pathways. Additionally, activated EGFR can translocate into the nucleus and either act as a transcription factor for genes such as *CCND1* or transcriptional coactivator for STAT and some miRNAs. *EGFR* is oncogenic in HNSCC, being amplified in up to 30 % of HNCs. The gene is also mutated and overexpressed in some HNCs. The elevated levels and mutations of *EGFR* in theses tumors can cause constitutive signaling via spontaneous monomeric receptor dimerization and tyrosine kinase activation. Co-expression of both receptor and ligand (TGFα) indicates it can cause autocrine signaling in some cancer cells. *EGFR* variant 3 mutant (*EGFRvIII*—lacks exons 2–7) is expressed in ~42 % of HNCs and is also associated with lack of response to anti-EGFR monoclonal antibody, cetuximab biotherapy. *EGFR* overexpression is a prognostic factor, conferring poor outcomes in HNC patients.

2.4.3.2 TGFβ Pathway Alterations in HNC

Transforming growth factor β (TGFβ) is a growth inhibitory signaling pathway associated with HNSCC. The TGFβ1 ligand interacts with TGFβ receptors to phosphorylate and activate SMAD2 and SMAD3, which then combine with SMAD4 to form the SMAD complex. The SMAD complex enters the nucleus to bind transcription factors, coactivators, or corepressors to control expression of TGFβ1 pathway target genes. Pathway activation is associated with decreased cell proliferation, decreased cell survival, and increased apoptosis. The *SMAD* and *TGFβRII* locus on chromosome 18q are frequently lost in HNC fields and contribute to invasive cancer formation. Additionally, TGFβ receptors are downregulated in HNCs.

2.4.3.3 PI3K/AKT Pathway Alterations in HNC

PIK3CA encodes p110α, a catalytic subunit for the PI3K class Ia molecules. The locus of *PIK3CA*, chromosome 3p26, is commonly gained or amplified in HNCs. Additionally, activating mutations in *PIK3CA* occur in 10–20 % of HNCs, especially HPV-negative tumors, and these cancers tend to have increased vascular invasion and lymph node metastasis. The PI3K pathway negative regulator, *PTEN*, is inactivated in ~10 % of HNCs as well.

2.4.3.4 *TP53* Alterations in HNC

Tumor protein p53 (*TP53*) and *retinoblastoma* (*RB*) are commonly mutated in HNSCC, and the proteins are targets of viral oncoproteins E6 and E7. These alterations lead to cell cycle deregulation, enabling cells to escape senescence and hence replicate uncontrollably. Besides its role in apoptosis, p53 controls the cell cycle at G2, following DNA synthesis at the S phase. P53 primarily functions to prevent expansion of unrepaired replicative errors, should they occur. In normal proliferating cells, p53 is usually targeted by MDM2 for ubiquitin-mediated proteasomal degradation, thus keeping the levels very low. In conditions of DNA damage, the p53 pathway is activated leading to increased expression and activity of p21CIP (*CDKN1A*), which stops cell cycle progression by negatively controlling the activity of cyclin/CDK complexes. Somatic mutations in *TP53* occur in 60–80 % of HNCs, mostly HPV-negative tumors. In HPV16-infected HNCs, the E6 oncoprotein targets and inactivates p53.

2.4.3.5 Cell Cycle Gene Alterations in HNC

Cell cycle progression through the G1 to S phase is controlled by RB tumor suppressor protein. In non-proliferating cells, RB binds and inactivates E2F transcription factor. In proliferating cells, activated cyclin D1/CDK4 and cyclin D1/CDK6 complexes phosphorylate RB leading to the release of E2F, which targets and induces the expression of genes including cyclin E for G1–S phase transition. Cyclin E/CDK2 complex further phosphorylates RB, driving cell cycle progression into the S phase. In order for senescence and cell differentiation to occur, p16 is expressed, and this inhibits the cyclin D1/CDK4 and cyclin D1/CDK6 complexes. *CCND1* is amplified or gained in over 80 % of HNSCCs and homozygous loss at chromosome 9p21, the *CDKN2A* locus is frequent in HNCs. Gene mutations and promoter hypermethylation also inactivate *CDKN2A*. HPV oncoprotein E7 targets the RB pocket proteins RB1, RBL1 (p107), and RBL2 (p130).

2.4.3.6 *MET* Alterations in HNC

Mesenchymal-epithelial transition factor (MET), located on chromosome 7p31, is a receptor for hepatocyte growth factor or scatter factor. It encodes an RTK that upon ligand interaction activates the PI3K and RAS-MAPK pathways. Mutations and amplifications of MET are found in HNSCC. MET activation is associated with increased cell growth, angiogenesis, and the metastatic phenotype.

2.4.3.7 Telomere Alterations in HNC

Another important alteration in HNC is telomere lengths. *Telomerase* (*TERT*) expression is increased in ~80 % of HNCs, but the *TERT* locus on chromosome 5p15.33 is not frequently gained or amplified in HNCs.

2.5 Circulating HNC Biomarkers

The clinical potential of ccfDNA and epigenetic alterations in ctDNA, miRNA, and serum proteins has been explored as HNC biomarkers. Circulating HNC cells demonstrate some utility in disease staging, prognosis, and treatment predictions. Validation of these liquid biopsy biomarkers will augment HNC management.

2.5.1 Circulating Cell-Free Nucleic Acids as HNC Biomarkers

Nucleic acid integrity is compromised in circulation of HNC patients, enabling their exploration for disease detection. For example, a study that employed multivariate analysis, plasma DNA integrity index (DII) was found to be significantly higher in patients with HNC than in controls. Optimal performance was obtained at a sensitivity of 84.5 % and a specificity of 83 % using a DII cutoff value of 0.82. But, this study failed to observe changes in postoperative samples as will be expected if the differences were due to cancer origin of high molecular weight DNA. The authors asserted to the possibility of residual population of cells with altered DNA degradation despite surgical removal of the cancer. Given the established importance of field cancerization in HNC, this conclusion is conceivable for this type of cancer. DII may only be useful for diagnostic purposes [4]. Chan et al. also examined DII before and after curative radiotherapy and compared them to controls without cancer [5]. They targeted *LEP* (leptin gene) fragments of lengths 105 bp and 201 bp. DII, defined as the ratio of 201 bp to 105 bp DNA fragments, was significantly higher in plasma from nasopharyngeal

carcinoma (NPC) patients than controls. After radiotherapy, DII was reduced in 70 % of the cancer patients. Patients with lack of reductions in DII had significantly poorer survival revealed by Kaplan–Meier analysis. Persistently high DII was associated with significant poor disease-free survival (DFS). Another investigation targeted RNA alterations in HNC, dubbed RNA integrity index (RII). Because of increased RNAase activity in circulation of cancer patients, reduced RNA integrity in plasma of cancer patients is expected. In this study, RII, defined as the ratio of 3′-to-5′ transcripts of *GAPDH*, was assayed as a diagnostic and predictive biomarker of patients with NPC. RII was significantly lower in plasma from patients with untreated NPC compared to healthy controls, and this ratio correlated with tumor stage. Interestingly, 74 % of the patients exhibited significant increased plasma RII after radiotherapy [6]. Thus, DII or RII shows potential as biomarkers of HNC, but the lack of specificity to this cancer, except when used in high-risk individuals, may hamper generalized utility.

2.5.2 Circulating HNC Epigenetic Biomarkers

Head and neck cancer is characterized by early epigenetic alterations (e.g., *CDKN2A* promoter hypermethylation). Several of these epigenetic changes have been explored in circulation, though disproportionately focused on NPC patients.

Methylation of *CDKN2A*, *MGMT*, *GSTP1*, and *DAPK* was assayed in primary HNCs and then in paired sera. Except *GSTP1*, 55 % of tumors had promoter hypermethylation in at least one of the genes. Fifty patients had paired sera, and methylation was demonstrated in 42 % of these samples. *DAPK* methylation was associated with lymph node metastasis and advanced disease state [7]. Methylated *DAPK* as a diagnostic biomarker in NPC has been assayed in tumor tissues, cell lines, plasma, and buffy coat samples. Hypermethylation was observed in 75 % of NPC tissues and 80 % of cell lines. Fifty percent (50 %) of plasma samples and 25 % of buffy coat samples harbored *DAPK* methylation, with 66.7 % of either sample being positive. *DAPK* methylation was not stage dependent and therefore can serve as an early detection biomarker [8]. Hypermethylation of *high in normal 1* (*HIN-1*) was also assayed in primary NPC tissues and matched nasopharyngeal swabs, throat rinses, and blood samples. Methylation in association with gene downregulation was present in all cell lines and 77 % of primary NPC samples. Methylation was in 46 %, 19 %, 18 %, and 46 % of nasopharyngeal swabs, throat rinses, plasma, and buffy coat, respectively [9]. This same group examined the possible use of gene methylation as screening and predictive biomarkers. Methylation of *CDH1*, *DAPK, CDKN2B*, *CDKN2A*, and *RASSF1A* was present in 71 % of plasma from NPC patients. Methylation of at least one of *CDH1*, *DAPK*, or *CDKN2A* was in 38 % of recurrences but none of the patients in remission [10]. The screening potential of *RIZ1* methylation has been demonstrated. *RIZ1* methylation in primary NPC tissues, cell lines, body fluids, and swabs was examined. Hypermethylation was present in cell lines, 60 % of primary NPCs, 37 % of

nasopharyngeal swabs, 30 % of mouth or throat washes, 23 % of plasma, and 10 % of buffy coat samples. *RIZ1* has a role for screening for NPC because all controls were negative (no false-positive results—100 % specificity as in many methylation assays) [11].

Global methylation studies have identified risk and possible early detection methylation patterns and biomarkers in circulation of patients with HNC [12, 13]. In a case–control study, Hsiung et al. reported that DNA hypomethylation in blood was significantly associated with elevated risk of HNSCC. A 1.6-fold risk was demonstrated even when controlled for other HNSCC risk factors. Smokers, individuals with low folate intake, and those with *MTHFR* genotypes had decreased global methylation and hence increased risk of cancer. In another genome-wide methylation profiling, six CpG islands methylated in circulation were most useful in HNC early detection. Methylation of *FGD4*, *SERPINF1*, *WDR39*, *IL27*, *HYAL2*, and *PLEKHA6* achieved an AUROCC of 0.73 (95 % CI 0.76–0.92) in HNC detection.

2.5.3 Circulating HNC Noncoding RNA Biomarkers

Oncomirs and tumor suppressormirs from HNC have been studied and multiple relevant targets identified. These miRNA and target deregulation, probably the tip of the iceberg at the moment, influence important signaling pathways involved in HNC progression.

2.5.3.1 Oncomirs and Tumor Suppressormirs in HNC

Overexpressed in HNC and with identified targets are miR-21, miR-17-92 polycistron, miR-181b, miR-106a, miR-106b-92, miR-106b-25 cluster, miR-155, miR-205, miR-221, and miR-345. The elevated level of miR-21 is associated with increased cell growth and apoptotic suppression via reduced cytochrome c release. These functions are achieved by suppression of a number of tumor suppressors including *PTEN*, *PDCD4*, *TPM1*, and *SERPINB5*. Indeed, overexpression of miRNAs including miR-21, miR-181b, and miR-345 drives leukoplakia toward invasive OSCC. In addition to miR-21, *PTEN* is a target of miR-205 in HNSCC.

Cell cycle deregulation is an important event in HNSCC progression. In addition to epigenetic and genetic alterations, cell cycle components are major alterations in head and neck carcinogenesis. For instance, miR-221 suppresses *CDKN1B* and *CDKN1C* mRNAs, while miR-17-92 and miR-106a target and degrade *RB1*. MiR-106a, 106b-25, and miR-17-92 clusters target CIP cyclin/CDK inhibitor, *CDKN1A*, that encodes p21. Consistently, knockdown of miR-106b-25 in HNSCC cells decreases cell proliferation via G1 phase arrest. Additionally, miR-106b-92 and miR-17-92 cluster interfere with TGFβ and MYC signaling pathway communication leading to cell cycle deregulation and loss of apoptotic

response. MiR-155 targets *APC* tumor suppressor gene and also controls TGF-β-mediated epithelial-to-mesenchymal transition by targeting *RHOA* transcripts.

Tumor suppressor miRNAs identified so far in HNSCC include let-7, miR-100, miR-125a/b, miR-133, and miR-200a. All let-7 family members (except let-7i) are downregulated in HNSCC, and these target *KRAS* and *HMGA2*. MiR-125a/b degrades *ERBB2*. Thus, the loss of miR-125a/b increases *ERBB2* expression associated with EGFR signaling in HNSCC. Downregulation of miR-100 in HNSCC leads to overexpression of oncogenes such as *ID1*, *FGFR1*, and *MMP13*. Pyruvate kinase M2 (PKM2), a well-known metabolic regulator in cancer cells, is a target of miR-133a/b. MiR-200a, which is downregulated in OSCC, targets E-cadherin repressors *ZEB1* and *ZEB2*, thus promoting epithelial-to-mesenchymal transition, tumor cell migration, and invasion.

2.5.3.2 Circulating HNC miRNA Biomarkers

A number of miRNAs have been examined in circulation of patients with HNSCC as diagnostic and prognostic biomarkers. Five circulating miRNAs, miR-16, let-7b, miR-338-3p, miR-223, and miR-29a, yielded an AUROCC of >0.80, suggesting their potential utility as noninvasive biomarkers for the detection of oral cancer or high-grade lesions [14]. MiR-17, miR-20a, miR-29c, and miR-223 were of diagnostic relevance for NPC [15]. Plasma miR-31 levels are much higher in OSCC patients than controls, and the levels decreased after surgical tumor removal. Also plasma miR-27b levels are reduced in oral cancer patients. In patients with oral cancer and precancerous lesions, plasma mR-196a and miR-196b levels were significantly higher than controls. While both demonstrated excellent performances independently, the combination of the two yielded an AUROCC of 0.845 for detection of precancerous lesions and 0.963 for oral cancer [16].

Circulating prognostic HNC biomarkers include miR-21, miR-26b, and miR-181. The expression of miR-181 is associated with progression of leukoplakia to invasive OSCC. Circulating and tissue miR-181 levels correlate with lymph node metastasis, invasiveness, and overall poor survival. This miRNA promotes cell migration and invasion [17]. MiR-21 (established to be markedly upregulated in cancer tissues) is significantly increased in plasma of patients as well. Plasma concentrations of miR-21 and miR-26b reduced postoperatively in patients with good prognosis but remained high even after surgery in those with poor outcome [18].

2.5.4 Circulating HNC Serum Protein Biomarkers

There are several altered proteins in circulation of HNC patients. Mostly ELISA has been used to evaluate this extensive number of serum biomarkers as diagnostic and prognostic biomarkers of HNSCC. However, only a few appear as being clinically

useful. The comprehensive meta-analytical synthesis by Guerra et al. [19] reveals the following:

- Majority of the studies involved single biomarkers, which expectedly are associated with dismal performances.
- Panel biomarkers had improved sensitivity and specificity for HNSCC detection.
- Thus, 34.3 % of panel and 12.8 % of single serum biomarkers examined were discriminatory for HNSCC.
- Commonly investigated serum biomarkers were CYFRA21-1, SCCA, and CEA.
- Of 15 single biomarkers, prolactin, catalase, glutathione, and β2-microglobin were of superior diagnostic performances for HNC. But β2-microglobin appears to be the most valid single biomarker with diagnostic potential.
- Panel biomarkers comprised of SCCA/EGFR/cyclin D1, and EGFR/cyclin D1 achieved acceptable diagnostic performances for HNC.

In addition to their diagnostic potential, several serum protein biomarkers are also associated with disease outcome and other clinicopathologic variables. For example, decreased overall survival is associated with elevated levels of CXCL9 (>209 pg/ml) [20], MMP (>226.7 ng/ml), and VEGF (>497.04 pg/ml) [21], while DCR3 (>284 pg/ml) is associated with nodal metastasis and poor prognosis [22].

Cheng et al. examined oral cancer plasma proteome using affinity bead-based protein purification, which involved the use of different chemical chromatographic surfaces with magnetic beads to discriminately select and purify certain subset of proteins of interest [23]. Bound proteins to the magnetic beads were eluted, purified, and diluted for MALDI-TOF MS analysis. With this approach, six spectral peak biomarkers differentiated patients with cancer from those without. A specific biomarker, a fragment of fibrinogen α-chain, was very accurate for oral cancer, achieving a sensitivity of 100 %, at a specificity of 97 %. Thus, proteomic approach may uncover additional high-performing discriminatory biomarkers for HNC.

2.5.5 Circulating HNC Cells

Consistent with many solid tumors, HNC patients tend to present with locally advanced (stages III/IV) disease. Indeed, ~10 % of patients present with distant organ involvement. These late diseases are treated with combined modalities including surgery, radiotherapy, and chemotherapy. Despite improved techniques in surgery and other treatment protocols, locoregional and distant recurrences are high, contributing to the dismal 40–50 % 5-year survival rates. Clinical approaches for detection of recurrences and distant metastasis rely on conventional imaging, which lack early detection capability. Invasive biopsy sampling of lesions for histopathologic examination is also required to confirm relapse, and this procedure is not possible in some cases. The need for “liquid biopsy,” a minimally invasive

blood sampling for circulating HNC cells (CHNCCs) to ascertain disease status, is obvious and has been explored. To this end, current efforts are focused on the use of CHNCCs for prognosis, early detection of micrometastasis, and monitoring of treatment response and other pertinent clinical applications.

2.5.5.1 Methodologies for CHNCC Analysis

Several methods and techniques have been employed for CHNCC enrichment, isolation, and detection. Many investigators have used the automated CELLSEARCH® CTC system that targets epithelial cell marker, EpCAM. One justification or rationale for the use of this system in CHNCC isolation and detection is the finding that in the HNSCC cell line, FaDu, the system was able to detect EpCAM and cytokeratin (CK) positive cells at significantly high levels (>95 % of these cells were positive) [24]. In 2002, Wirtschafter et al. used monoclonal antibodies against tumor antigens including EpCAM to isolate CHNCCs [25]. This assay successfully detected CHNCCs in 44 % of the samples. An obvious issue with this method is the fact that not all CHNCCs express EpCAM or CK. Cytokeratin (*KRT*) may be downregulated with tumor progression and invasion. Loss of *EPCAM* and *KRT* expression is much more common in tumor stem cell-like cells undergoing EMT. Indeed, a subset of highly aggressive breast cancer cells does not express *EPCAM*. To overcome such issues, an immunomagnetic enrichment followed by RT-PCR targeting expression of multiple surrogate markers including *KRT19*, *ELF3*, *EGFR*, and *EPHB4* enabled the detection of CHNCCs at a high frequency of 87.5 % [26].

2.5.5.2 CHNCCs as Staging Biomarkers

The frequency of positive CHNCC detection is TNM stage dependent. Tumor size and depth of invasion are correlated with the rate of CHNCC detection. The higher the T status, the higher the frequency of positive CHNCCs. Similarly, CHNCCs were detected at a higher rate in T3–T4 (48.8 %) than T1–T2 (31.7 %) tumors [27]. Another trend was the detection of 94 CHNCCs/ml of blood in patients without gross disease, compared to 193 CHNCCs/ml in those with gross disease. While T1 tumors had no detectable CHNCCs, 17 % of T2–T4 tumors were positive for circulating tumor cells. CHNCCs in advanced stage disease were significantly associated with pulmonary involvement [24]. Some of these differences are not statistically significant; however, they demonstrate a trend for increased number of CHNCCs in advanced stage disease [28]. Nodal status also has positive correlations with CHNCC detection. CHNCCs were recovered at 61 % frequency in N2b or higher compared to 21 % of N0-2a patients. This finding was significant in multivariate analysis [29]. Of eight patients positive for CHNCCs, only one was N0, with the remaining being N1-2a [30]. Again there is a positive trend for CHNCC detection with increasing nodal involvement. CHNCCs are detected at a

lower frequency (<20 %) in stage I/II disease than the frequency of >40 % in advanced stage III/IV tumors. Consistent with these findings, multiple studies associate CHNCC presence with increasing tumor stage.

2.5.5.3 CHNCCs as Prognostic Biomarkers

CHNCCs in relation to disease-free survival (DFS), progression-free survival (PFS), and overall survival (OS), as well as other survival metrics, have been evaluated. The study by Wollenberg et al. indicated that positive CHNCCs at primary therapy were associated with increased risk of disease recurrence and metastasis [27]. Similarly, the risk of local failure and distant metastatic disease was evident in patients with circulating CK19-positive cells [31]. CHNCCs, when present, were associated with increased probability of disease recurrence [32]. The detection of CHNCCs before surgery predicted worse DFS. Survival outcome was worse in patients with high levels of CHNCCs [33]. Nichols et al. also detected better survival in patients without CHNCCs [24]. The presence of over two lymph node involvement, as well as CHNCCs detected by targeting E48 mRNA, conferred poor distant metastasis-free survival [34]. In another series, 50 % of CHNCC-positive patients had recurrences compared to 27 % of patients without detectable CHNCCs. CHNCC-positivity was associated with worse OS [35]. The evidence so far supports a role for CHNCC characterization as prognostic biomarkers of clinical relevance.

2.5.5.4 CHNCCs as Predictive Biomarkers

Biomarkers that can predict response to chemotherapy, radiotherapy, biotherapy and other therapeutic interventions should enable personalized treatment choices to be made. This could potentially spare responders the unnecessary surgery, and nonresponders the undesired toxicities of the therapeutic intervention. Serial monitoring for treatment response will benefit from biomarker analysis in body fluids. For example, some patients initially respond to anti-EGFR biotherapy, but efficacy changes as a result of mutations in members of the pathway in some evolving clones detectable in circulation. Simple blood analysis of CHNCCs should enable treatment adjustments to be made to these individuals.

Reversal of CHNCCs to null following chemotherapy and radiotherapy was indicative of complete or partial response. The lack of CHNCC detection during treatment was also indicative of nonprogressive disease [28]. Activated EGFR pathway in CHNCCs (detected by targeting phosphorylated EGFR) was present in ~55 % of CHNCCs. Radiotherapy in combination with cetuximab therapy was more effective than radiotherapy and cisplatin/5-fluorouracil chemotherapy at reducing the numbers of these pEGFR+ CHNCCs [36]. The detection of CHNCCs correlated with regional metastasis in patients with inoperable HNSCC, and the

frequency reduced by concurrent radiotherapy and chemotherapy but was present in 20 % of patients during treatment [29].

2.5.5.5 Induction of CHNCCs by Clinical Interventions

Evidence indicates clinical procedures can increase the number of CHNCCs, and this has biologic implications. Systemic spread of CHNCCs has been detected following biopsy or surgery for HNC, and radiotherapy was also associated with increased numbers of CHNCCs [33, 36–38]. However, without going through the metastatic program to acquire the relevant cues needed for distant implantation, it is unclear whether these cells have the potential of forming metastatic deposits. More research is needed to address this important question.

2.5.6 Circulating HPV-Positive HNC Biomarkers

While the incidence of HPV-negative HNSCC is on the decline, the reverse is true for HPV-positive cases. The two etiologies are also molecularly and clinically different. For instance, the prognosis is better for HPV-positive HNC, indicating the ability to identify these cases should lead to less intense treatments and thus reduce the toxicity and other treatment-associated adverse effects on these patients. However, there are no consensus diagnostic criteria for this subset of HNCs. Diagnostic approaches rely on analyses of tissue samples for HPV DNA and RNA by PCR and in situ hybridization, coupled with immunohistochemistry for p16 protein expression. A promising noninvasive approach is the detection of HPV in saliva or oral rinses.

The analysis of serum HPV-specific IgG as a diagnostic biomarker of HPV-positive HNSCC has been explored. Mork et al. performed a nested case–control study within the joint Nordic cohort, where blood samples were available from nearly 900,000 people [39]. After 9.4 years follow-up, 292 developed HNSCC, and together with 1568 matched controls, antibodies against HPV16, 18, 33, and 73 were analyzed. After adjusting for cotinine levels (marker of smoking status), the odd for cancer was 2.2 in patients positive for anti-HPV16 antibodies (and not the other subtypes). Because oral sex is a risky lifestyle for HPV infections, the presence of serum anti-HPV immunoglobulin was associated with an elevated risk for HNSCC among HIV-positive individuals [40]. Specifically, antibody levels against HPV16 proteins (E1, E2, and E7) strongly correlated with OPSCC [41]. As a prognostic biomarker, circulating antibodies against HPV16 E6 and E7 proteins are favorably associated with all-cause survival in OPSCC patients [42]. Seropositivity appears to correlate with viral load and disease stage.

2.5.7 HNC Extracellular Vesicles

Tumor-derived circulating extracellular vesicles (EVs) are present in patients with HNSCC. These EVs play a role in tumor escape from cell death and possibly other tumor biology. Thus, they contain factors of immunosuppressive and apoptotic effects on activated T cells. This extracellular vesicular content can cause pan-caspase activity on CD8+ Jurkat cells, possibly through the presence of FasL on the vesicles. They induce apoptosis via both receptor-mediated and mitochondrial pathways. These effects are more pronounced in serum EVs derived from advanced stage active disease patients [43].

2.6 Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is endemic in Southeast Asia and Southern China, with increasing incidences in Northern Africa, the Mediterranean, and among the Inuits of Alaska. Estimated incidence rate in Hong Kong of about 20/100,000 contrasts sharply with the rest of the world with age-adjusted incidence rate of under 1/100,000. This geographic variation is the consequence of the complex interplay between environmental and genetic factors. Indeed, Chinese born in China retain the same elevated risk level irrespective of which part of the world they migrate to, compared to the relatively low risk in, for example, Chinese born in North America.

The histopathologic classification of NPC is based on the degree of tumor differentiation. The WHO identifies three subtypes. Type I tumors are highly differentiated keratin-producing squamous cell carcinomas with characteristic epithelial growth patterns. Types II and III are undifferentiated non-keratinizing tumors. Type II tumors differ from type III only by retaining their epithelial cell shape and growth architecture. A simpler and etiologically more relevant classification dichotomizes NPCs into SCC (similar to WHO type I tumors) and undifferentiated carcinomas of the nasopharyngeal type (UCNT, WHO type II and III tumors).

Type I (SCC) tumors are the least aggressive and are the prevalent cancers in the Western World, representing ~75 % of all cases. This contrasts with those from Southern China that are mostly (>97 %) the UCNT. In addition to their aggressive nature, UNCT tumors are strongly associated with EBV infection, and this infection is an etiologic factor in NPC progression. Although elevated risk factors for NPC include high salt consumption during childhood, this appears to require additional genetic factors such as expression of some HLA haplotypes, as well as alterations at susceptibility loci including those on chromosomes 3p21, 3p26, 4p15.1-q12, 9q21, and 13q12. Molecular pathology of NPCs includes high-frequency alterations on chromosomes 3p, 9, 12, and 14q, some of which are associated with known cancer susceptibility genes such as *CDKN2A* (9p21) and *RASSF1A* (3p21.3).

Hypermethylation and inactivation of *CDNK2A* and *RASSF1A* are very common in NPCs. The multistep pathogenesis of NPC includes initial genetic alterations followed by EBV infection that exerts its oncogenic properties to drive the nasopharyngeal cell into becoming an invasive cancer.

2.6.1 Circulating EBV DNA in NPC Patients

Nonoyama et al. in 1975 described circulating EBV DNA (CEBV DNA) in NPC patients [44]. This was a couple of years before the exploration of ccfDNA as cancer biomarkers. A body of evidence establishes the clinical utility of circulating viral genomes, especially in NPC patients. Also, circulating HPV genomes appear relevant in cervical cancer and possibly in HPV-positive HNSCC.

Circulating biomarkers for EBV detection have been explored since the early 1970s. Serological tests, targeting viral antigens such as early antigens and viral capsid proteins (EAs, CVA, EBNA1, ZEBRA, and EBV DNase), have been developed for use as diagnostic tests for EBV-mediated NPC. While these immunofluorescent and ELISA tests are still available, a more robust genetic assay was pursued and has been optimized for use in the management of NPC caused by EBV infections. This is a qPCR assay targeting CEBV DNA. Because WHO type I NPCs harbor negligible EBV genomes, these tests target the types II and III (UCNT) NPCs, which constitute the majority of those in endemic areas such as Asia.

2.6.1.1 Circulating EBV DNA Quantification

The development of molecular genetic assays for EBV detection had to overcome some challenges:

- An issue that needed to be resolved in order to improve test sensitivity was to understand the genomic structure of EBV and hence targets to select for qPCR assay development. Infection with EBV is ubiquitous in endemic areas, so EBV specificity to tumor was critical to assay specificity. It was uncovered that the expression of *EBER1* and other genes is restricted to NPC [45, 46]. In many healthy people, the immune system efficiently eliminates viral particles. But in NPC cells, EBVs thrive, such that detectable circulating EBV DNA is a specific indication of NPC. Even then, positive detection rate of EBV DNA is 2–4 % among healthy people.
- Although EBV infection and residence in NPC cells exhibit type II latency, with restricted expression of viral genes such as *LMP1*, *LMP2*, *EBNA1*, *EBERs*, and *BamH-A*, theoretically all viral genomes can be targeted in circulation. However, given the inherent biomarker dilution effect of circulating plasma, analytical detection sensitivity increases by targeting viral genes with increased copy numbers or sequences with multiple repeats. Viral genome structural analyses

indicate the suitability of targeting three viral genes, namely, *EBNA1*, *EBER1*, and *BamH-W*. Both *BamH-W* and *EBNA1* harbor multiple repeats, while *EBER1* exhibits copy number increases in infected NPC cells.

- The detection sensitivity of CEBV DNA also depends on whether serum or plasma is used for testing. There are reported differences in CEBV DNA qPCR test accuracies, especially sensitivity in regard to whether plasma or serum is the test sample. The reported sensitivity has been higher for plasma (90–99 %) than serum (68–86 %), but they both retain high specificity of 87–100 % for plasma and 89–100 % for serum samples.
- Finally, the size of amplified DNA influences detection positivity. Naked circulating EBV DNA is highly fragmented, with molecules mostly <80 bp in circulation [47]. Therefore, the sensitivity of detection, which was initially low (31–75 %) because of targeting large amplicons (168–293 bp), has now been greatly improved by recent targeting of smaller (59–76 bp) DNA fragments.

2.6.1.2 Clinical Utility of CEBV DNA

The work by Lo et al. in 1999 suggested a high degree of association of circulating EBV DNA with NPC [48]. While present in 96 % of patients, the positive rate was only 7 % in healthy individuals. Moreover, the levels of CEBV DNA were associated with tumor burden and hence stage dependent. Experimental mouse models further buttressed the elevated levels in advanced stage disease. CEBV DNA assays are currently in use for NPC screening, diagnosis, prognosis, as well as treatment and recurrence monitoring in endemic areas.

CEBV DNA as a Diagnostic Biomarker of NPC

For a new test to replace or favorably compete with existing test, it must outperform or show some favorable characteristics absent in the old or gold standard assay. Thus, for general adoption of CEBV DNA assay in NPC screening and diagnosis, it had to be compared to the serological IgA-VCA immunofluorescent (IF) and ELISA assays. For plasma testing, the mean sensitivity and specificity of CEBV DNA test are 94 % each. While the sensitivity of IgA-VCA test is a little low (mean 87 %), the specificity suffers from having a wider range (46–96 %), as well as being low (mean 76 %). The diagnostic performance of plasma CEBV DNA test with sensitivity of 90–99 % and specificity of 87–100 % thus appears much superior to IgA-VCA assays.

The sensitivity and specificity of serology were, however, elevated with two-step ELISA tests for EBNA1 and VCA followed by a confirmatory assay with IgA-EA test. This approach gave improved sensitivity of 97 % and specificity of 98 %. Similarly, VCA detection by ELISA appears to have similar accuracies to CEBV DNA assay, as revealed by a meta-analysis of 20 studies [49]. The sensitivity and specificity are 91 % and 92 %, respectively, from this analysis. Therefore these two serologic approaches appear useful for NPC diagnosis as well.

The test performance is dependent on the number of CEBV DNAs. The median levels of EBV DNA to enable early detection are 6000 genomes/ml, and this is maintained by the release of three million genomes per hour into the circulation [48]. Thus, the sensitivity of the test also depends on the stage of the disease, because mean viral load of 2500 copies/ml in stage I disease patients contrasts with the high loads of 32,590, 86,000 and 166,2000 gene copies/ml in stages II, III, and IV disease patients, respectively [50]. Thus, the detected sensitivities of CEBV DNA tests of 50–86 %, 94–95 %, 91–100 %, and 94–98 %, respectively, for stage I, II, III, and IV are consistent with the designated viral loads.

CEBV DNA as a Prognostic Biomarker of NPC
The EBV serologic assays have demonstrated no prognostic utility, as they are not associated with outcome variables in NPC patients. However, CEBV DNA tests are very accurate in NPC management. Both high pretreatment and posttreatment viral loads are associated with poor survival [51, 52]. In one series, a set cutoff of EBV DNA load of 1500 gene copies/ml was accurate at survival prediction [53]. Of clinical relevance were the findings that CEBV DNA levels could stratify early stage I and II disease patients into good and poor prognostic groups, which is important for guiding treatment decision-making [51].

CEBV DNA as a Treatment and Recurrence Monitoring Biomarker of NPC
The circulating levels of EBV DNA show good dynamic response to surgery, radiotherapy, and chemoradiotherapy. It has been established that the levels significantly fall following treatment, except for patients with recurrent diseases. The test positive frequency in those without recurrences is 0–12 %. This percentage contrasts with those with recurrences, where 63–90 % remain positive. Indeed, the more aggressive chemoradiotherapy treatment of NPC reduces DNA levels more dramatically than only radiotherapy. Similarly, nasopharyngectomy is associated with reduced viral DNA positive frequencies down to 0–29 % of cases.

Following an initial drop in treatment, the subsequent increases in CEBV DNA load indicate disease relapse, and this may precede clinical evidence of disease progression [48, 54 55]. The kinetics of CEBV DNA has revealed a rapid clearance from the circulation. To et al. demonstrated a median short half-life of 138 min following surgery [56]. Therefore, an elevated viral DNA load of 500 copies/ml or higher detected 6 weeks following treatment is indicative of relapse [57]. These patients require further investigations such as PET/CT for disease localization.

2.6.2 Circulating NPC Exosomes

Nasopharyngeal cancer cells shed exosomes into the tumor microenvironment that eventually enter body fluids such as saliva and the peripheral circulation, the two media in which they were first described. In addition to the usual exosomal cargo of oncogenes including nucleic acids, proteins, and lipids, exosomes from EBV-infected NPCs are enriched with EBV-encoded miRNAs, membrane-

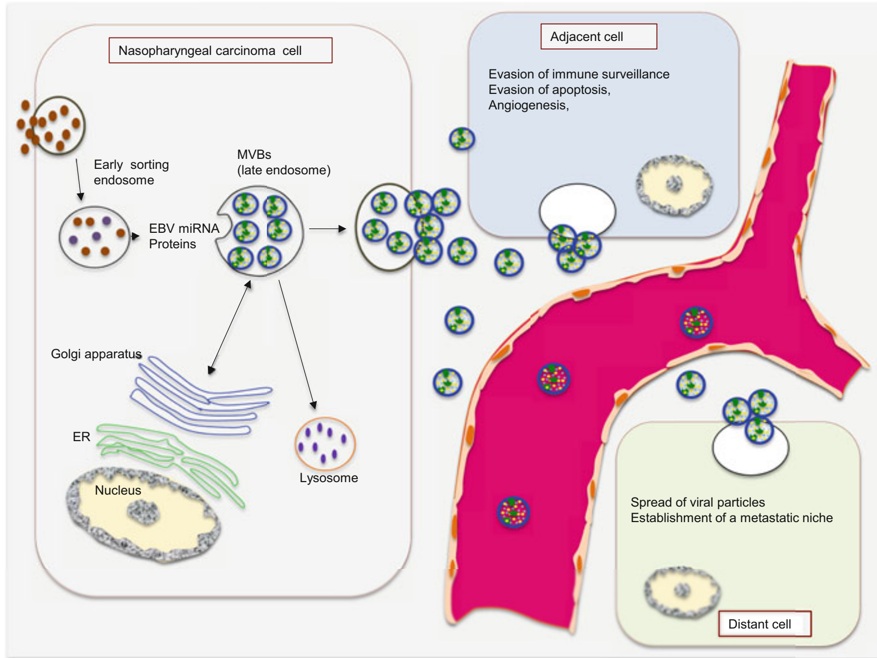

Fig. 2.5 EBV exosomes in NPC and their effects on adjacent and distant cells. *ER* endoplasmic reticulum, *MVB* multivesicular bodies

associated oncogenic factors such as latent membrane protein 1 (LMP1) and galectin 9, as well as molecules involved in signal transduction pathways such as EGF and FGF. These exosomal contents are implicated in myriads of cancer-related functions including evasion of immune surveillance and apoptosis, angiogenesis, spread of viral particles, and possible preparation and establishing the metastatic niche for implantation (Fig. 2.5).

2.6.2.1 Exosomal LMP1 in NPC

Latent membrane protein 1 is a primary EBV-encoded oncoprotein that is a member of the TNFR family. It is involved in tumor initiation, progression, and lymphangiogenesis and contributes to metastasis by promoting epithelial-to-mesenchymal transition, cell motility, and invasion. These effects are partly achieved by the fact that LMP1 regulates multiple signaling pathways including PI3K, MAPK, JNK, and NF-κB. LMP1 is detectable in serum exosomes from NPC patients. The effects of LMP1 are dose dependent. Low levels can favor tumor development; however, high levels inhibit tumor cell growth and sensitization to apoptosis. Interestingly, EBV-miR-BART targets LMP1 and, importantly, is considered to "fine-tune" the levels of LMP1 that are conducive for NPC progression.

LMP1 also regulates host cell miRNA expression. For example, LMP1 increases the expression of miR-146a that targets and destroys IFN-responsive genes and hence favors escape from immune surveillance.

2.6.2.2 Exosomal Galectin 9 in NPC

Galectin 9 is a β-galactosyl-binding lectin with immunosuppressive functions. It has multiple roles in tumor biology including tumor cell adhesion, survival, immune escape, and angiogenesis. Galectin 9 suppresses both T and B lymphocytes and specifically induces apoptosis in T cells by interacting with TIM1 membrane receptor. In NPC exosomes, galectin 9 is contained in HLA class II-positive exosomes, together with LMP1. The use of anti-HLA class II immunomagnetic capture technique enables isolation of NPC-specific exosomes from patient plasma that contains high levels of galectin 9. Their presence in circulation could contribute to immunosuppression. Thus, their functional biology, and use as biomarkers of NPC, requires further investigation. They may also serve as therapeutic targets.

2.6.3 EBV-Encoded miRNAs in NPC

EBV is the first human virus uncovered to express its own miRNAs [58]. The EBV-miRNAs are organized into two main clusters: (i) the *BamH1-H open reading frame 1* (*BHRF1*) and (ii) the *BamH1-A rightward/region transcripts* (*BARTs*). The *BHRF1* cluster encodes three miRNA precursors (EBV-miR-BHRF1-1, EBV-miR-BHRF1-2, and EBV-miR-BHRF1-3) that are processed into four mature miRNAs. The largest cluster, *BART*, transcribes 22 precursor miRNAs that give rise to 44 mature miRNAs. The *BART* miRNAs are more commonly found in exosomes and are expressed by type II latency viral program (*BHRF1* mainly type III latency). The importance of EBV-miRNA is further suggested by the fact that a considerable proportion (~15 %) of total miRNAs in NPC cells is of BART origin.

2.6.3.1 EBV-miRNA Targets in NPC

The EBV-encoded miRNAs are involved in multiple oncogenic processes including evasion of host immune surveillance, evasion of apoptosis, and alteration of several signaling pathways. This is achieved through targeting several members of these signaling pathways (Table 2.1). Cytotoxic T lymphocytes within the microenvironment of NPC cells lack cytotoxic activity and are unable to produce IFNγ, despite extensive infiltration of these tumors by leukocytes (CD4+, CD8+, B cells, NK cells, and macrophages). The reason for the severe immune impairment within the tumor cell microenvironment is partly due to the following numerous functions of EBV-encoded miRNAs.

Table 2.1 EBV-miRNAs and their targets in NPC

EBV-miRNA	Targets
	Host cell targets
	Apoptosis
EBV-miR-BART cluster 1 and 2	*BIM* (*BCL2L11*)
EBV-miR-BART 5	*PUMA*
EBV-miR-BART 16	*TOMM22*
	Immune
EBV-miR-BHRF 1-3	*CXCL11/i-Tac*
EBV-miR-BART 6-5p	*DICER*
EBV-miR-BART 2-5p	*MICB*
EBV-miR-BART 3-3p	*IPO7*
	WNT signaling
EBV-miR-BART 19-3p	*WIF1*
EBV-miR-BART 7, EBV-miR-BART 19-3p, EBV-miR-BART 17-5p	*APC*
EBV-miR-BART17-5p	*NKD*
	Viral targets
EBV-miR-BART 2-5p	*BALF5*
EBV-miR-BART 22	*LMP2A*
EBV-miR-BART 1-5p, EBV-miR-BART 16, EBV-miR-BART 17-5p	*LMP1*

Evasion of Immunosurveillance

Evasion of the immune system is mediated by the following:

- BART2-5p targets and destroys MHC class I-related chain B (MICB) in EBV-infected NPCs. NKG2D is a potent activating receptor on NK cells and also is a costimulatory receptor on γδT and CD8+ T cells. MICB is a cellular ligand for NKG2D used by NK and T cells to eliminate viral infected and neoplastic cells. Thus, reduced expression of MICB enables evasion of the host immune system.
- Additionally, EBV-miR BART2-5p interacts with the 3′ UTR complementary sequences of viral lytic gene *BALF5* leading to mRNA degradation. *BALF5* is a viral DNA polymerase involved in EBV replication. Destruction thus interferes with viral replication and hence maintenance of viral latency. This prevents viral exit into the lytic replicative phase where immunogenic molecules are expressed that can easily be recognized by the immune system.
- Importin 7 (IPO7) is a nuclear import receptor that heterodimerizes with importin-β to shuttle signaling molecules and transcription factors into the nucleus. Nuclear targets of IPO7 include NFAT, a transcription factor involved in cytokine gene expression in activated T cells. In EBV-infected NPC microenvironment, *EBV-miR-BART3* is transmitted to tumor-infiltrating lymphocytes via exosomes to mediate suppression of IPO7 expression, leading to decreased cytokine production by such T cells.
- Dicer is a target of *EBV-miR-BART6*. The destruction of Dicer in EBV-infected NPCs causes not only defects in global miRNA biogenesis but maintenance of

viral latency and decreased expression of potent immunogenic latent proteins such as EBNA2 and LMP1.

- Another highly immunogenic viral latent gene, LMP2A, is a target of *EBV-miR-BART22*. The interaction with 3′ UTR sequences and destruction of LMP2A leads to decreased protein levels, preventing immunologic recognition and destruction of EBV-infected NPCs.

Evasion of Apoptosis

Antiapoptotic activities of these miRNAs are established by:

- The 3′ UTR of pro-apoptotic gene BIM is a target of BART cluster 1 and 2 EBV-miRNAs. Lack of BIM proteins suppresses p53-dependent apoptosis.
- *EBV-miR-BART5* suppresses pro-apoptotic gene, p53 upregulated modulator of apoptosis (PUMA). Consistent with this finding, PUMA levels are reduced in ~60 % of NPCs, and depletion of *EBV-miR-BART5* increases apoptosis in EBV-positive NPC cells.
- TOM22 is a mitochondrial receptor for BAX that mediate BAX-dependent mitochondrial apoptosis. *EBV-miRBART16* targets and destroys TOM22.
- The CXCL11/iTac cytokine gene is a target of *BHRF1-3*.
- BART 7, BART 19-3p, and BART17-5p target APC.
- Secretion of T cell inhibitory NPC exosomes.

2.6.4 Clinical Potential of Circulating EBV Targets in NPCs

EBV targets have been measured in the peripheral circulation as potential adjuncts to disease detection and management. LMP1 and BART1 viral oncoproteins were detected in serum and saliva from NPC patients [59]. Exosomal BART miRNAs were also stably assayed in plasma from both NPC xenografts and NPC patients [60]. The levels of EBV-miR-BART-2-5p, EBV-miR-BART-6-5p, and EBV-miR-BART-7-5p are much higher in sera from NPC patients than controls, and these levels significantly correlate with the elevated expression in primary tumors [61].

2.6.5 Summary

- HNCs are a heterogeneous group of neoplasms with distinct etiologies, epidemiology, and molecular genetic drivers.
- HNSCC is dichotomized into HPV-positive and HPV-negative subgroups. Not only are they etiologically different, but also the molecular pathogenesis is equally distinct. For example, while both exploit *TP53* alterations and cell cycle deregulation in their pathogenesis, HPV-negative tumors harbor *TP53* mutations, in contrast to wild-type *TP53* in HPV-positive tumors. However, HPV-positive tumors degrade and inactivate p53 using viral oncoprotein E6.

- A third and distinct subset of HNC is NPC, with the primary etiologic agent being EBV infection.
- The molecular and genetic aberrations that characterize all these cancers are reflected in body fluids as well, and have been pursued as clinically actionable biomarkers. For example, circulating EBV biomolecules have been developed as routine laboratory tests for NPC in endemic areas.

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Chapter 3
Lung Cancer Biomarkers in Circulation

Key Topics

- Issues with lung cancer (LnCa) screening
- Molecular pathology of LnCa
- Circulating cell-free nucleic acid content as LnCa biomarkers
- Circulating LnCa epigenetic biomarkers
- Circulating LnCa genetic biomarkers
- Circulating LnCa miRNA biomarkers
- Circulating LnCa protein biomarkers
- Circulating LnCa cells

Key Points

- The potential for early detection of LnCa can be realized given the well-charted molecular pathology and subtypes. This expansive knowledge on LnCa molecular genetics is also propelling the development of effective targeted therapies for the various molecular subtypes.
- Extensive work has culminated in the discovery of a plethora of circulating LnCa biomarkers with profound clinical potential. Traditional serum biomarkers are still of important clinical utility in LnCa management. However, targeting molecular imprints in circulation, such as Resolution Bio ctDx™ Lung assay, enables the liquid biopsy paradigm to be realized.
- Circulating LnCa cells are used for disease prognosis and treatment monitoring and have potential for mutation detection (e.g., *EGFR* mutations) for real-time therapeutic decision-making.

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3.1 Introduction

For several decades, LnCa has remained a challenge in oncology by being the highest in both incidence and prevalence. The global incidence for 2012 and 5-year prevalence were 1.825 million and 1.893 million, respectively. Sadly, of the 1.825 million cases, 58 % occurred in the resource-poor parts of the world. Added to this unfavorable statistics are the dismal survival rates. An estimated 1.59 million global deaths were expected for 2012, and again many (963,000) occurred in the developing world. The prevalence of LnCa in the USA alone is at 430,090, and the estimated incidence in 2016 is 224,390, with 158,080 expected deaths. Globally, LnCa is the leading cause of cancer-related deaths. Even more worrisome is the fact that the global incident rate mirrors the mortality rate, which is partly due to the high case fatality coupled with the lack of regional variability in survival rates. This dismal statistics of LnCa is due to a number of reasons among which are:

- The lack of evidence-based screening modalities (imaging or biochemical markers), even for those at elevated risk above the general population, such as smokers.
- Because of the lack of recommended screening guidelines, the vast majority of patients are diagnosed with late and advanced stage disease, when the prognosis is poor. If detected early, the 5-year survival rate can be as high as 70 %. However, this rate is ~20 % for stage IIIb and 5 % for stage IV LnCa. Improved imaging and treatment has enabled some progress to be made over the past several decades in the 5-year survival rates.
- Patients with late stage disease are subjected to chemotherapy and various targeted therapies. But lack of response and evolution of resistant clones lead to relapse. Additionally, field cancerization accounts for some relapse due to the emergence of second primary tumors.
- Although LnCa is histopathologically dichotomized into non-small cell LnCa (NSCLC, 80 % of all cases) and small cell LnCa (SCLC, the remaining 20 %), the disease is very heterogeneous at the molecular level, thus, hampering efficient targeted therapy delivery.

Clearly, early detection of LnCa is key to improving the dismal 5-year survival outcome. The need for validated biomarkers and molecular imaging techniques that can be deployed at the point of care is urgent. Of even more importance will be biomarkers that can be assayed noninvasively in body fluids for LnCa screening, diagnosis, treatment stratification, longitudinal monitoring for treatment response, detection of early recurrences, and for the study of tumor evolution over time so as to inform clinical decision-making. Thus, various investigators are diligently pursuing these biomarkers and this chapter provides a synthesis of these findings.

3.2 Screening Recommendations for LnCa

Why has LnCa early detection and possible chemoprevention been a problem when it appears to be the easiest and straightforward cancer to pickup early when treatment is optimal? The answer may appear simple but is more complicated than can be appreciated, due probably to the issues to overcome before implementation of screening programs.

Because of the lack of evidence that screening reduces mortality, LnCa screening is not recommended even for high-risk individuals. Historically, LnCa screening had relied on chest X-ray (CXR), sputum cytology, and recently low-dose spiral CT (LDCT) scans of the lung. Chest X-ray screenings are not good for early LnCa detection because they pick up lesions that are 2 cm or larger. They also have low specificity to differentiate between mimickers of cancer such as sarcoidosis, pulmonary tuberculosis, and other lung infections from LnCa. Sputum cytology is an established diagnostic approach for LnCa, but it lacks sensitivity, especially for early detection, because frank cancer cells are rarely found in sputum from patients with early stage disease.

The first published prospective study on LnCa screening using CXR was in 1968. In this large cohort study, it was concluded that annual mortality rate was substantially similar between screened and unscreened populations, although the 5-year survival rate was much higher among the screened population. There have been five randomized control trials published between 1984 and 2011. These are the Mayo Lung Project (MLP), Johns Hopkins Lung Project (JHLP), Memorial Sloan Kettering Lung Project (MSKLP), Czechoslovakia Study, and recently the Prostate, Lung, Colorectal, and Ovarian (PLCO) screening trial. The consistent findings from all these studies are that:

- LnCa detection rate is similar between the study and control groups.
- Early stage tumors are detected in screened populations (except for the MSKLP study).
- No difference in overall survival or disease-free mortality is observed between the groups (except that the 5-year survival rate was better in the screened group in the MLP study).
- Long-term (20 years) follow-up revealed no difference in disease-specific mortality between the screened and controlled groups.
- The PLCO study of 1993 randomized 154,901 people between the ages of 55–74, who either had CXR annually for 3 years or received conventional care. This study also concluded that CXR with or without sputum analysis does not reduce disease-specific mortality.

The MLP study showed that CXR or CXR combined with sputum cytology enabled the detection of early stage cancers and increased patient survival but had no effect on reducing mortality. A confounding variable with this study was the increased detection of insignificant lesions, resulting in the problem of

overdiagnosis. Thus, CXR with or without sputum cytology does not appear to reduce disease-specific mortality in high-risk populations.

Computerized tomography scans are becoming very sensitive for LnCa early detection with improved scanner resolutions. They can even detect nodules as small as 1 mm, but the problem is that not all nodules are cancerous. This increased sensitivity has led to increased detection of benign nodules (~95 % of all nodules are benign), thus making it costly, aside from exposing patients to unnecessary radiation as well as causing increased patient anxiety. While CT is associated with high false positives and costs, low-dose spiral CT (LDCT) has shown promise as a screening modality for LnCa, especially in high-risk populations. Multiple large uncontrolled studies indicate that LDCT detects early stage I, small tumors with favorable 5-year survival outcomes. Bach et al. reviewed three uncontrolled trials (Moffitt Cancer Centre, Tampa, Fl; Mayo Clinic; and Istituto Tumori in Milan studies) involving 3246 asymptomatic at-risk individuals [1]. The conclusion was that LDCT significantly detected more LnCas than expected, including many patients with early stage I/II cancers (78 % of cases). Disappointingly though, this screening did not significantly reduce mortality rates as expected, and the number of advanced stage LnCas diagnosed did not decrease in the screened population. However, guidelines by the National Comprehensive Cancer Network (NCCN) for LnCa screening recommends an annual LDCT scanning for high-risk individuals between the ages of 55–74 who quit smoking 15 years or earlier with at least 30-pack years of smoking history. Multiple randomized control trials are in progress with initial published data from the National Lung Screening Trial (NLST) study indicating a 20 % relative reduction in LnCa-specific deaths among high-risk individuals being attributable to LDCT screening compared to CXR [2]. While recommended by the NCCN for screening high-risk individuals 55–74 years of age, LDCT is costly and not universally available or accessible.

Bronchoscopy allows direct visualization and even biopsy of lung lesions. However, this method is invasive and expensive and only scans the major airways. It will therefore miss up to 40 % of all LnCas located in the periphery. In the face of all these evidences, screening decisions are varied and rest on the physician and patient. Tobacco avoidance or cessation still remains the first-line preventive measure of reducing the societal burden of LnCa. However, recent efforts on clinical utility of biomarkers could change the dismal outlook on screening. LnCa is a disease of epigenetics and genetics, and hence several alterations in biomolecules are potential clinical useful biomarkers. These epigenetic and genetic changes impinge on cellular regulatory pathways involved in apoptosis, proliferation, and senescence. Aberrant effects of oncogenes and tumor suppressor genes primarily mediate the development of both SCLC and NSCLC. Validated assays targeting these biomarkers noninvasively should permit implementation of acceptable screening programs for at least the high-risk population.

3.3 Molecular Pathology of LnCa

From a clinical and molecular pathologic perspective, primary LnCa is classified into SCLC and NSCLC (Table 3.1). NSCLC, which accounts for the majority (80 %), is a broad category of LnCas that are nonresponsive to SCLC treatment regimes. This group is further subdivided into adenocarcinoma (accounts for >50 % of LnCas), squamous cell carcinoma (SCC), and large cell carcinoma. SCLC tends to be aggressive and metastatic, and hence is often treated by chemotherapy. NSCLC, however, is less aggressive and is amenable to surgery. Neuroectodermal tumors include SCLC, large cell neuroectodermal carcinoma (LCNEC), and carcinoid tumors. These are very aggressive tumors with neuroendocrine morphology. They therefore express at least one of the following neuroendocrine markers: synaptophysin, chromogranin, or CD56. The three subgroups differ primarily in their level of mitosis and the presence or absence of necrosis.

LnCa is a conglomerate of heterogeneous diseases, and this complex or diverse histology reflects on the molecular pathology as well. Important signaling pathways with multiple gene alterations on LnCa include the PI3K, RAS-MAPK, JAK/STAT, among others. Multiple oncogenes (*KRAS*, *EGFR*, *BRAF*, *MEK1*, *HER2*, *MET*, *ALK*, *RET*) and tumor suppressor genes (*TP53*, *PTEN*, *and LKB-1*)

Table 3.1 Pathologic classification of LnCa

Tumor (frequency)	Pathologic features
NSCLC (~80 %)	
Adenocarcinoma	Glandular differentiation with or without mucin
Squamous cell carcinoma	Squamous differentiation with keratinization
Large cell carcinoma	Diagnosis of exclusion. Classified as NSCLC-NOS; can have squamous or adenomatous differentiation; diagnosed on surgically resected samples
Adenosquamous carcinoma	At least 10 % of both squamous and glandular differentiation. Definite diagnosis on surgically resected samples
Sarcomatoid carcinoma	Poorly differentiated NSCLC with features of sarcoma or sarcoma-like; spindle and/or giant-cell carcinoma
Carcinoma of salivary gland type	Mucoepidermoid, adenoid cystic, or epithelial-myoepithelial forms
Neuroendocrine tumors (20–25 %)	
SCLC	Small tumor cells, scant cytoplasm, fine granular chromatin, no evident nucleoli; necrotic with high mitotic rate (≥11 mitotic figures/10 high-power field (HPF)
Large cell neuroendocrine carcinoma	Cytologically resemble NSCLC but with neuroendocrine morphology and positive for at least one NE marker; necrotic with high mitotic rate (≥11 mitotic figures/10 HPF
Carcinoid	Common in children; neuroendocrine morphology; typical carcinoid tumors have no evidence of necrosis and low mitotic rate (<2 mitoses/10 HPF); atypical forms have focal necrosis and intermediate mitotic rate (2–10 mitoses/10 HPF)

are activated or silenced by diverse mechanisms and at various frequencies and patterns in LnCa.

3.3.1 Genetic Alterations in LnCa

Chromosomal structural and numeric instabilities are common in LnCa. These include nonreciprocal translocations and deletions involving tumor suppressor genes, amplifications of oncogenes, and aneuploidy. Allelic losses at 3p, 4p, 4q, 5q, 8p, 10q, 13q, 17p, and 22q are common in SCLC, while losses at 3p, 6q, 8p, 9p, 13p, 17p, and 19q are associated with NSCLC. Microsatellite instability (MSI) is observed in ~35 % of SCLC and ~22 % of NSCLC. Efforts to use MSI in early detection of LnCa using body fluids and sputum have been encouraging. Here are synopses of some genes altered in LnCa.

3.3.1.1 *TP53* Alterations in LnCa

TP53 is inactivated in a significant proportion of LnCas and is one of the major genetic alterations in this disease. For example, LOH at 17p13, the *TP53* locus is observed in ~90 % of SCLC and ~65 % of NSCLC. Inactivating mutations, mostly involving the DNA-binding domain occurs in 80–100 % of SCLC and in ~47 % of NSCLC. The Cancer Genome Atlas estimates 81 % mutation frequency in SCLC. Additionally, ~45 % of adenocarcinomas harbor these *TP53* mutations, which are tobacco smoke carcinogen induced. Consistently, the mutations are higher in smoking-associated G to T than G to C transversions. Never smokers often harbor G to A transitions in these cancers. These mutations are associated with treatment resistance and poor prognosis.

3.3.1.2 *KRAS* Alterations in LnCa

The *KRAS* oncogene is frequently mutated in LnCa, primarily NSCLC of the adenocarcinoma histology. The mutations are virtually absent in SCC and SCLC. *KRAS* driver mutation frequencies vary geographically and with sex and lifestyle exposures. Thus, it appears to be more common in Western populations than Asians and more frequent in males and smokers of both sexes. As a member of the RAS-MAPK pathway, at least one component of this pathway is mutated in ~70 % of tumors, with most mutations clustered in the *KRAS*. Recorded mutation frequency varies from 25 to 40 % in adenocarcinomas. The identified hotspot mutation is a single amino acid substitution in codon 12, but also rarely in codons 13 and 61. In smokers, G to T transversions occur at a much higher frequency of ~84 %. Tumors from never smokers rather tend to harbor G to A transitions. *KRAS* mutations are mutually exclusive in tumors with *EGFR* mutations, and tumors with

these mutations confer resistance to EGFR-TKI therapy because of constitutive pathway activation downstream of EGFR. *KRAS* mutations have prognostic value, and this appears to vary with the types of mutation as well. Data from the Biomarker-Integrated Approaches of Targeted Therapy for LnCa Elimination (BATTLE) indicate that G12C or G12V mutant *KRAS* is associated with shorter progression-free survival than other mutations or wildtype genotype.

3.3.1.3 *EGFR* Alterations in LnCa

Similar to *KRAS*, almost all *EGFR* mutations occur in adenocarcinomas and rarely in adenosquamous carcinomas. They are almost exclusive in SCCs, but *EGFRvIII* mutations that alter the extracellular domain of the receptor, as well as copy number gains and increased receptor expressions, are more common in SCC than adenocarcinomas. *EGFR* mutations are more common in tumors from younger people, females, and nonsmokers. Mutation prevalence also varies geographically, being 30–40 % in Asians compared to 10–15 % in people from the Western world. Activating mutations are clustered in the first four exons that encode the intracellular tyrosine kinase domain. Most are inframe deletions in exon 19 (45 %). Of the over 20 reported deletions, del E746-A750 is the commonest in NSCLC. Missense L858R mutation in exon 21 is the next frequently observed mutation (40 %). In early stage cancer, exon 18 mutations can account for 14 % of *EGFR* mutations, while L858R comprises about 29 %. Inframe duplications and insertions in exon 20 that often confer resistance to EGFR-TKIs account for 5–10 % of *EGFR* mutations in LnCa. However, the commonest EGFR-TKI resistant mutation is the activating point mutation c.2369C>T (T790M) in exon 20, which accounts for 50 % of all resistant mutations. The T790M substitution interferes with the binding efficiency of TKIs. Usually these mutations develop during treatment and are clonally selected for. However, exon 20 mutations including T790M have been found in patients who had no previous treatments.

3.3.1.4 *BRAF* Alterations in LnCa

Activating mutations in *BRAF* that increase kinase activity occur in ~3 % of NSCLCs, mostly adenocarcinomas. Other mutations affect the G-loop of the activation domain. Kinase domain mutations include V600E, D594G, and L596R, while activating domain mutations include G465V and G468A. About half of all mutations in adenocarcinomas are accounted for by the common V600E, followed by G469A and D594G. V600E mutation is common in female nonsmokers, while the other mutations are more frequent in smokers than never smokers. Mutations are also mutually exclusive of other pathway gene (e.g., *KRAS*, *EGFR*) mutations.

3.3.1.5 *MET* Alterations in LnCa

MET oncogene is amplified in at least 1–7 % of NSCLC. Copy number increase is more common in SCC than adenocarcinoma where they occur in 3–5 % of cases. MET amplification leads to overexpression, constitutive receptor phosphorylation, and activation of downstream pathways including PI3K and RAS-MAPK. MET amplification accounts for ~20 % of resistance to EGFR-TKIs through signaling via the PI3K pathway, thus obviating the need for EGFR signaling.

3.3.1.6 *HER2* Alterations in LnCa

Human epidermal growth factor receptor 2 (*HER2*) encodes membrane bound receptor tyrosine kinase, a member of the ERBB/EGFR receptor family. Although HER2 does not bind to ligands, it can heterodimerize with other ligand-bound family members to signal via the PI3K, MAPK, and JAK/STAT pathways. Activating mutations occur in up to 4 % of NSCLC. Gene amplifications occur in 2 % of NSCLC, but increased expression is demonstrated in as many as 20 % of NSCLC. Exon 20 inframe insertions of 3–12 bases are the common activating mutations in *HER2*. These alterations are common in adenocarcinomas and are mutually exclusive of *EGFR* and *KRAS* mutations. *HER2* alterations are also associated with nonsmokers, people of Asian descent, and female gender.

3.3.1.7 PI3K Pathway Alterations in LnCa

PI3K is an important signaling pathway in LnCa, being altered in 50–70 % of NSCLC, especially SCCs that demonstrate a mutation frequency of 47 % (according to the Cancer Genome Atlas Project). Activating mutations in *EGFR*, *KRAS*, *AKT*, *PI3K*, and *PIK3CA*, as well as loss of *PTEN*, are altered pathway components. Mutations and amplifications in *PIK3CA* cause constitutive pathway activation. Mutations, mainly in the catalytic domain, are observed in 1–3 % of NSCLC, and increased copy number is observed in ~5 % of SCLC. *PIK3CA* mutations may occur in association with *KRAS* and *EGFR* mutations. *AKT* mutations occur in 0.5–2 % of NSCLC, especially SCC. *PTEN* mutations occur in ~5 % of NSCLC (more in SCCs than adenocarcinomas; in ~10 % SCC compared with ~2 % of adenocarcinomas). Transcriptional repression of *PTEN* with decreased protein levels is more common in NSCLC (~75 %).

3.3.1.8 Alterations of Cell Cycle Regulators in LnCa

RB1 was the first described tumor suppressor gene in LnCa, and this gene is activated in a vast majority of LnCas (~90 % of SCLC and 10–15 % of NSCLC).

Inactivation of *CDKN2A*, a negative regulator of the cell cycle, occurs in ~80 % of NSCLC, especially SCCs in which alterations occur in ~72 % of cases. *CCND1* overexpression is observed in ~40 % of NSCLC.

3.3.1.9 *FGFR1* and *DDR2* Alterations in LnCa

Membrane-associated receptor tyrosine kinase FGFR1 signals via MAPK and PI3K pathways. *FGFR1* amplifications are demonstrated in ~20 % of SCCs (uncommon in adenocarcinomas). Membrane-associated receptor tyrosine kinase *DDR2* is mutated in ~4 % of SCCs.

3.3.1.10 *LKB-1/STK11* Alterations in LnCa

LKB encodes serine-threonine kinase that inhibits mTOR and mTOR pathway components (excluding KRAS). *LKB-1* inactivation via mutations and deletions alone occur in 11–30 % of adenocarcinomas. Alterations are associated with smoking and male gender and correlate with *KRAS* mutations.

3.3.1.11 Fusion Genes in LnCa

While gene fusions are often more of a feature of hematologic malignancies, recent advancements indicate their presence in some solid tumors as well. Thus, subsets of LnCas harbor *RET*, *ALK*, and *ROSI* fusion rearrangements.

RET encodes receptor tyrosine kinase that is normally involved in neural crest cell development. Alterations are common in papillary and medullary thyroid cancers. *RET* activation through chromosomal rearrangement is however observed in some LnCas. The functional kinase domain (exons 12–20) are fused to *kinesin family 5B* (*KIF5B*) that is 10 Mb from the *RET* locus (10q11.2). *KIF5B-RET* fusion occurs in 1–6 % of LnCas, primarily adenocarcinomas from never smokers. This activated fusion gene is mutually exclusive of other LnCa driver mutations including *KRAS*, *HER2*, *EGFR*, *ALK*, *BRAF*, and *ROSI*.

Activation of ALK receptor tyrosine kinase signals through the PI3K, RAS-MAPK, and JAK/STAT pathways. *ALK* is activated by chromosomal rearrangements when fused to *echinoderm microtubule-associated protein-like 4* (*EML4*). Various variants with different lengths of *EML4* have been identified. Fusions involve inversion in chromosome 2p; the commonest is intron 13 of *EML4* fusion to intron 19 of *ALK*. Other reported fusion partners are *KIF5B*, *TRK-fused gene* (TFG), and *Kinesin light chain 1* (*KLC-1*). Rearrangements occur in ~4 % of NSCLC, especially in adenocarcinomas from young nonsmokers.

ROSI activation signals via the PI3K, RAS-MAPK, and STAT3 pathways. It encodes membrane-bound tyrosine kinase receptor that is homologous to ALK kinase domain. Multiple 5′ fusion partners have been identified in 1–3 % of

LnCas, mainly adenocarcinomas from younger people, nonsmokers, and people of Asian descent (similar to *ALK*). Fusion partners include *TPM3*, *CD74*, *E2R*, *SLC34AZ/NaPi2b*, *FIG*, *KDELR2*, *SDC4*, and *LRIG3*. However, oncogenic functions of these partners are yet to be determined.

3.3.2 Molecular Classification of LnCa and Personalized Medicine

From the standpoint of unbiased and accurate classification that informs treatment selection, molecular phenotyping of LnCa is desirable. Several gene expression profiling, mutation detection, and other genetic approaches provide strong evidence for the molecular heterogeneous nature of LnCa. Thus, from the perspective of actionable oncologic practice, LnCa is viewed from the molecular profile.

Targeted therapies are mainly focused on the larger group of NSCLC, because the molecular pathology of this histologic subgroup has been fairly characterized. In regard to pharmaceutical development, biotherapies are targeted mostly at altered cancer "driver genes," mainly oncogenes and tumor suppressor genes. Given experiences with evolving resistance to monotherapies, it is likely that combinatorial therapies may be a more useful approach. For example, the T760M mutation in *EGFR* causes resistance to erlotinib and gefitinib, while *ALK* C1156Y and L1196M mutations render cancer cells resistant to crizotinib. Furthermore, as sequencing costs become more affordable, it will be possible to sequence tumors at a depth and resolution that will enable tumor heterogeneity to be identified more accurately. This should inform the use of therapies targeting all clones (even the very occult ones) in tumors. In NSCLC, a few targeted genes have been identified. It should be noted that the molecular underpinnings of many LnCas (e.g., over 40 % of adenocarcinomas) are still elusive. Well-established alterations in lung adenocarcinoma are *KRAS*, *EGFR*, *NRAS*, *BRAF*, *ALK*, *ROS1*, *RET*, *HER2*, *AKR1*, *PIK3CA*, and *MAP2K* and in SCC are *EGFR*, *EGFRvIII*, *DDR2*, *PIK3CA*, and amplifications in *FGFR1* [3]. Targeted therapies are either available or being developed for several members of these pathways (Table 3.2). For example, EGFR-TKIs, gefitinib and erlotinib target tumors with exons 18–21 mutations. Similarly, ALK-TKI, crizotinib targets *ALK* translocations. With the advent of personalized medicine, targeted molecular therapy is imperative for all cancers. Hence, for LnCa, the IASLC/CAP/AMP recommends that all lung adenocarcinomas be tested for *EGFR* mutations and *ALK* translocations.

Table 3.2 Molecular targeted therapies for lung adenocarcinoma

Targets	Agents
ALK	Crizotinib
BRAF	Vemurafenib, GSK2118436
EGFR	Afatinib, erlotinib, gefitinib
FGFR1	Brivanib, ponatinib, AZD4547, S49076
HER2	Afatinib, dacomitinib, neratinib
MEK	AZD6244
MET	Onartuzumab, rilotumumab, cabozantinib, crizotinib, tivantinib
PD-1/PD-L1	Nivolumab, MPDL3280A
PIK3CA	GDC-0941, XL-147, BKM120
PTEN	Vandetanib
RET	Cabozantinib, sunitinib, sorafenib, vandetanib
ROS1	Crizotinib
NSLC34AZ/NaPi2b	DNIB0600A

3.3.3 Multistep Carcinogenesis and Field Cancerization in LnCa

LnCa develops in two stepwise fashions, depending on whether it is SCC or adenocarcinoma. The sequence of events for bronchial tumors is *hyperplasia–dysplasia–carcinoma in situ–invasive squamous cell carcinomas*, and for adenocarcinoma of peripheral bronchioles and alveolar, the progression pathway is *atypical alveolar hyperplasia–adenomatous and alveolar hyperplasia–adenocarcinoma*.

Auerbach and colleagues elegantly demonstrated the tobacco-induced lung field defect in 1961 [4], and several molecular events preceding LnCa have been demonstrated thereafter. Their findings are consistent with tobacco-related histological changes in lung epithelia, with the development of widespread multifocal premalignant lesions. Because smoking is a known risk factor for development of LnCa, studies have established a molecular field defect in smokers with or without cancer. In addition to other carcinogens, toxins from tobacco smoke are established agents that create the LnCa field, either directly or through induction of epithelial inflammation. About 85 % of smokers are at risk for developing LnCa (that is having established LnCa fields evidenced by molecular alterations), but only ~15 % will actually progress to develop the disease. Smoking causes aberrant promoter methylation of multiple genes in the epithelium of cancer-free individuals. These genes include *CDKN2A*, *DAPK*, *GSTP1*, *RAR-β2*, *RASSF1A*, *CDH13*, and *APC*. Thus, smokers have multiple foci of "damaged" epithelium with epigenetic alterations. Many lungs from smokers without cancer demonstrate numerous genetic changes as well, including loss of heterozygosity (LOH) and microsatellite alterations. Even in bronchial washes from both ipsilateral and contralateral lungs from smokers, LOH and *TP53* mutations are present. Additionally, in histologically

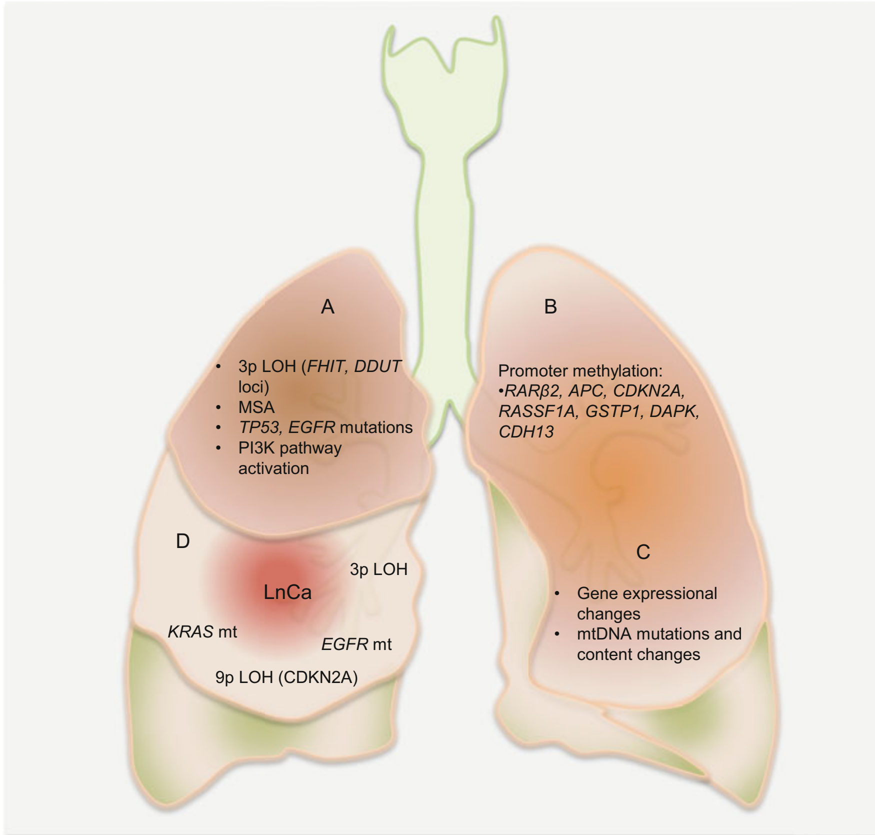

Fig. 3.1 Molecular pathology of lung field cancerization. In lung exposed to tobacco smoke without cancer (A, B, C), LOH, MSA, promoter methylation of tumor suppressor genes, mutations, gene expressional changes, mtDNA mutations, and activation of the PI3K pathway characterize the field. In normal appearing lung tissue close to LnCa (**D**), similar alterations are present

normal lung tissue adjacent to LnCa, mutations in *EGFR* and *KRAS*, as well as LOH at chromosome 3p (*DDUT* and *FHIT* loci) and chromosome 9p (*CDKN2A* loci), have been characterized. Figure 3.1 is a summary of the molecular alterations in tobacco smoke-associated pulmonary field cancerization. While this is the tip of the iceberg for LnCa, molecular field cancerization occurs in all tumors.

3.4 Circulating LnCa Biomarkers

Given the inherent problems with LnCa screening, there is a need for noninvasive biomarkers for this disease. Thus, circulating biomarkers have been extensively explored for LnCa management.

3.4.1 Circulating Cell-Free Nucleic Acid Content as LnCa Biomarkers

The clinical relevance of circulating cell-free DNA (ccfDNA) and RNA (ccfRNA) has been extensively evaluated in LnCa patients. Both quantitative analyses as diagnostic and prognostic biomarkers and more specific qualitative studies of epigenetic and genetic alterations in key genes involved in LnCa have been pursued. Various targets including *hTERT*, *ACTB*, *HBB*, and ALU sequences have been used to measure the amounts of circulating free DNA. While the findings are compelling and have potential clinical applications, the nonspecific nature of circulating cell-free nucleic acid (ccfNA) levels discourages the clinical translation of these findings. While gene mutations have been targeted qualitatively, it is also possible with advanced technologies to quantify the levels of mutations and methylations.

3.4.1.1 Cell-Free Nucleic Acid Content as Diagnostic Biomarkers of LnCa

The clinical applications of ccfDNA have diagnostic and prognostic relevance for LnCa. Sozzi et al. demonstrated the diagnostic and prognostic potential of quantitative plasma DNA in NSCLC patients. Mean plasma DNA levels were higher in patients, including those with early stage disease. The diagnostic accuracy achieved an AUROCC of 0.844. Microsatellite alterations and DNA levels correlated with clinical status and early detection of recurrence [5]. In a follow-up study, Sozzi et al. could discriminate NSCLC patients from matched controls for age, sex, and smoking status based on the levels of ccfDNA [6]. They amplified *hTERT* gene as a measure of ccfDNA in LnCa patients. In this well-designed study, median DNA concentration in patients was eight times that of controls, achieving a diagnostic accuracy with AUROCC of 0.94. Plasma DNA concentration was a strong risk factor for NSCLC. These findings have been confirmed by numerous other studies. The amount of ccfDNA from LnCa patients (~13 ng/ml of plasma) is significantly different from age and sex matched control individuals without cancer (~3 ng/ml of plasma). The diagnostic performance of ccfDNA measurement by Paci et al. achieved an AUROCC of 0.79 for NSCLC detection. Additionally, high levels of ccfDNA had prognostic value on follow-up [7]. While another study by Sozzi and colleagues found that baseline plasma ccfDNA levels did not improve the accuracy of LnCa detection by spiral CT in smokers, it was evident that higher levels of plasma ccfDNA at surgery still conferred elevated risk for the presence of an aggressive tumor [8]. Benlloch et al. found that median ccfDNA concentration was higher in both pleural effusions and sera from LnCa patients compared to controls. Additionally, higher ccfDNA in either fluid compartment correlated with poor survival [9].

Circulating cell surface-bound DNA (csb-DNA) as useful diagnostic biomarkers of LnCa has also been explored. While the amounts of ccfDNA were nondiscriminatory between cancer and controls, there was a significantly lower level of csb-DNA in cancer patients compared to controls. Consistent with other findings, these low csb-DNA levels correlated with poor survival [10].

The diagnostic potential of ccfDNA may not be of much utility as a stand-alone test for detection of LnCa; however, it could improve the diagnostic accuracy when combined with other tests, imaging assessments, and probably other clinical parameters in a nomogram.

3.4.1.2 Cell-Free Nucleic Acid Content as Prognostic Biomarkers of LnCa

It has been fairly established that the levels of ccfDNA have prognostic utility in LnCa management. High levels are indicative of aggressive disease and predictive of poor overall survival. Reduced levels of csb-DNA are also associated with poor prognosis. In multivariate analysis, plasma and serum ccfDNA have been found to be independent prognostic biomarkers in NSCLC patients [8, 9, 11, 12]. A high serum level of ccfDNA measured by targeting *hTERT* was an independent predictor of time to progression and OS of advanced stage NSCLC patients in multivariate analysis [13]. Circulating nucleosomal DNA is also much higher in cancer than healthy people.

While they may have limited use in cancer detection because of increased release in nonmalignant conditions such as trauma, stroke, inflammation, autoimmune diseases, and infection, ccfDNA and nucleosomal DNA levels have shown promise as prognostic biomarkers in LnCa. For example, Holdenrieder et al. found that nucleosomal DNA levels before first and second cycle chemotherapy were independent prognostic factors in multivariate analysis [14]. Circulating nucleosomal DNA is also useful for monitoring treatment response in LnCa. Patients who responded to treatment demonstrated decreasing circulating nucleosomal DNA levels, while nonresponders with progressive disease showed persistently high or increasing levels [15]. In several studies of advanced stage NSCLC patients on either first- or second-line chemotherapy, and also in SCLC patients on first-line chemotherapy, reducing levels or the absolute serum levels of nucleosomal DNA at staging investigation significantly predicted responders from nonresponders [16, 17]. Thus, plasma DNA dynamics appear useful for disease prognosis and monitoring for treatment efficacy or resistance.

3.4.2 Circulating LnCa Epigenetic Biomarkers

Epigenetic biomarkers are detected in tissue, sputum, and blood samples from LnCa patients. Commonly methylated is *CDKN2A*, which is detectable in almost

all LnCa tissue, 63 % of sputum, and 33 % of serum samples. Also, *MGMT* and *APC* methylation are found in 66 % and 50 % of serum samples, respectively. Low levels of *MGMT* and *APC* methylation are associated with better survival, and similarly, loss of *DAPK* and *RASSF1A* through methylation has prognostic implications. Hypomethylation and loss of imprinting of *IGF2* and *H19* are also features of LnCa.

Several a priori LnCa biomarkers are assayed in plasma and serum samples from patients, and various methylation frequencies have been demonstrated. The list includes methylation of *CDKN2A*, *GSTP1*, *DAPK*, *MGMT*, *RASSF1A*, *RAR-β*, *CDH1*, *CDH13*, *APC*, and *TMS1*, which are assayed singly or in combination as panels. In a proof of concept study, methylation status of four genes (*CDKN2A*, *GSTP1*, *DAPK*, and *MGMT*) was analyzed in serum samples from patients with NSCLC. Methylation of at least one gene was detected in 68 % of tumor samples and 73 % of paired serum samples. Methylation was specific, as it was not detected in normal lung tissue and tumor-negative patient serum samples. All stages of cancer harbored methylation in these genes [18]. *CDKN2A* promoter methylation was assessed in 111 tumor tissue samples, 136 plasma, and 95 sputum samples using semi-nested methylation-specific PCR (MSP). Methylation was positive in a majority of the tumor (80.2 %), plasma (75.7 %), and sputum (74.7 %) samples. When *CDKN2A* methylation in sputum and plasma was combined with sputum cytology, most (92 %) of the LnCas could be detected [19]. *CDKN2A* methylation in paired NSCLC tissue and plasma were assayed using a modified semi-nested MSP, which increased detection in both tissue and plasma samples. Methylation was in 79.3 % of tumor samples, of which 64 of the 73 positive cases (87.7 %) were demonstrated in paired plasma samples [20].

The prevalence of methylation in multiple genes was examined in plasma and sputum from women who were at various risks (cancer survivors, cancer-free smokers, and never smokers) for LnCa to determine their possible use as screening biomarkers. Methylation of three genes in plasma and seven genes in sputum was positive in LnCa survivors. LnCa survivors had the highest prevalence of *CDKN2A* promoter hypermethylation in plasma, as well as a significant increase in the odds (OR, 3.6) of having one or more methylated genes in plasma than never smokers [21]. NSCLC tissue and paired plasma samples were investigated for *CDKN2A* promoter hypermethylation, MSA at 3p, *KRAS* mutations, and ccfDNA concentration. Individually, these biomarkers demonstrated diagnostic potential. Methylation was detected in 63 % of tissue and 55 % of plasma samples. MSA was present in 57 % of tissue and 50 % of plasma samples, and *KRAS* mutations were detected in 31 % of tissue but undetectable in plasma samples. The combination of MSA and methylation status increased the sensitivity of this plasma diagnostic assay to 62 %, which was further increased to 80 % with the incorporation of ccfDNA levels, demonstrating the obvious advantage of multi-panel assays [22]. Methylation of *CDKN2A*, *MGMT*, *RASSF1A*, and *DAPK* in sera from 200 patients undergoing bronchoscopic evaluation due to abnormal chest radiographs was investigated. Methylation was more frequent in LnCa patients than patients with nonmalignant lung diseases. The diagnostic sensitivity and specificity of this serum assay were

49.5 % and 85 %, respectively. The odds of having LnCa when one gene was methylated in serum were 5.28, and this increased to 5.89 when two or more genes were involved. Noteworthy, 50.9 % of stage I disease patients had genes methylated compared to other serum protein markers that were positive in only 11.3 %, underscoring the importance of methylation biomarkers in early detection [23]. *CDKN2A* and *CDH13* methylation status in tissue and matched serum samples from 61 NSCLC patients was assayed with fluorescent MSP. Methylation frequencies in tissue were 79 % for *CDKN2A*, 66 % for *CDH13*, and 52 % for tumors harboring both gene methylations. Methylation in at least one gene was detectable in 92 % of tumor samples. For serum samples, the detection rate was 26 % for *CDKN2A*, 23 % for *CDH13*, and 39 % for at least one methylated gene. This study also demonstrated the diagnostic specificity because samples from healthy people were all negative [24]. Ninety-six percent of primary LnCa samples harbored *APC* promoter hypermethylation, of which 47 % of available serum/plasma samples were positive. No prognostic use was found for serum targets, but high levels of methylation in tumor tissue were independent predictors of poor survival, and detection of methylation in serum, however, was of diagnostic potential [25].

Gene promoter hypermethylation in circulation has also been associated with prognostic features of LnCa patients. The promoter methylation of the G2-M checkpoint control gene, *SFN* (14-3-3σ), was assayed in pretreatment sera as possible prognostic factor in advanced stage NSCLC patients on platinum-based chemotherapy. Methylation was positive in 34 % of serum samples from patients, and this was associated with longer median survival time. Multivariate analysis identified methylation of *SFN* as a novel (apart from Eastern Cooperative Oncology Group performance status) independent prognostic factor for patients with advanced stage NSCLC [26]. Methylation of multiple genes (*MGMT*, *CDKN2A*, *RARβ2*, *RASSF1A*, *FHT*, *CDKN2A/ARF*, *APC1A*, *APC1B*, *CDH1*, and *DAPK*) in sera from 46 LnCa patients was investigated using a nested MSP. All except *APC1B* showed various frequencies of methylation. The most frequently methylated genes were *APC1A* (78.1 %), *CDKN2A* (41.3 %), and *RASSF1A* (40.9 %). Methylation in at least one of these genes was present in 78.3 % of the patients and was associated with advanced tumor stage, size, and undifferentiated status [27]. Methylation of *RASSF1A*, *DAPK*, and *target of methylation-induced silencing* (*TMS*) as well as *KRAS* mutations was assayed in sera from 50 resected NSCLC patients. Tumor methylation status correlated significantly ($p = 0.001$) with serum frequencies (both ranged from 34 to 45 % for the assayed genes). Twelve *KRAS* mutations were found in serum samples, and these correlated significantly with survival (compared to methylation that had no prognostic utility in this study) [28]. *CDKN2A* methylation was detected in 42 % of NSCLC tissue samples and in only 14 % of plasma samples from patients with advanced TNM stage tumor. However, the presence of *CDKN2A* methylation in tissue and circulation was associated with poor survival and shorter disease-free survival. Pre- and post-resection pleural lavage fluids were positive for *CDKN2A* methylation that was equally associated with poor survival [29].

3.4.3 Circulating LnCa Genetic Biomarkers

The genetic alterations in LnCa hold promise as clinically actionable liquid biopsy biomarkers. Mutations in key genes and microsatellite alterations have all been successfully demonstrated in circulation of LnCa patients.

3.4.3.1 Circulating Mutated Genes as LnCa Biomarkers

Clinical utility of LnCa DNA measurements include, early detection in high-risk patients, intermediate end points in chemotherapy and other treatment trials, monitoring treatment efficacy, and as prognostic biomarkers for monitoring disease recurrence or disease-free intervals. In LnCa, studies of circulating genetic alterations have focused mainly on *KRAS*, *EGFR*, and *TP53* mutations. Because mutations in *KRAS* are associated with lack of response to EGFR TKIs, longitudinal profiling of *KRAS* alterations in circulation should enable accurate decision-making on patient management. Activating *EGFR* mutations enhance response to TKI treatment of NSCLC, while *KRAS* mutations may confer poor prognosis as well.

Sorenson et al. first detected mutated *RAS* in circulation of cancer patients in the 1990s [30]. However Catells et al. first demonstrated the diagnostic and prognostic potential of circulating *KRAS* mutations in cancer patients [31]. Gautschi et al. also asserted to the prognostic utility *KRAS* mutations in plasma of NSCLC patients [32]. Other investigators, however, failed to find prognostic utility for circulating *KRAS* mutations in LnCa patients. *KRAS* codon 12 mutations were detected in 30 % of patient plasma samples. The most common change was glycine to cysteine (90 %), but this mutation had no prognostic or predictive value [33]. A follow-up study of *KRAS* codon 12 mutations in advanced stage NSCLC patients similarly failed to uncover any prognostic relevance for this mutation [34].

Kimura et al. examined the potential of using *KRAS* mutations in treatment monitoring [35]. This study examined tissue and paired plasma samples from patients who were on carboplatin and paclitaxel chemotherapy. *KRAS* mutations in plasma were associated with poor survival (median 11.4 months in patients with wild type compared with dismal 3.3 months in those with mutant *KRAS*). It could be argued that the difference in months is irrelevant, but if detected early, in conjunction with other mutations that offer therapy alternatives, survival could potentially be prolonged. Plasma *KRAS* mutations were identical to those found in tissues. Following treatment, *KRAS* mutations were undetectable in posttreatment plasma of responders. This suggests its important utility in therapy monitoring. While Pao et al. found that *KRAS* mutations lacked sensitivity to predict response to kinase inhibitors, it was suggested that treatment decisions might be improved by analyzing both *KRAS* and *EGFR* mutational status [36].

Missense mutations in exon 21 and exon 19 of *EGFR* determine response to gefitinib. Several groups have been able to detect this mutation in ccfDNA from LnCa patients [37–39]. *EGFR* mutation was detected in 20 % of plasma samples

and was associated with clinical response to erlotinib and docetaxel treatment, as well as improved progression-free survival. *KRAS* mutations were, however, associated with rapid progressive disease [40]. *EGFR* exons 19 and 20 mutations were examined in plasma and matched tissue samples. Mutations were found in 34.5 % of samples. Clinically, these mutations were associated with increased response to gefitinib and significantly longer progression-free survival in advanced stage NSCLC [39]. *EGFR* T790M mutation confers resistance to TKIs. In one cohort of patients, both activating and T790M mutations were found in 70 % of ccfDNA and proved useful in monitoring resistance to gefitinib or erlotinib therapies in NSCLC patients [41].

A microfluidic digital PCR that performs 18 PCRs simultaneously was used to quantify exon 19 deletions and L858R mutations in *EGFR* [37]. These two mutations account for >85 % of *EGFR* mutations associated with response to TKIs. Exon 19 deletion and L858R mutations were detected in 17 % and 26 % of pretreatment plasma samples, respectively, and these mutations were accurate when tissue mutations were compared (sensitivity of 92 % and 100 % specificity). Decreased concentration of mutant sequences was associated with partial or complete remission.

TP53 is very susceptible to carcinogen damage. Smoking as a risk factor of lung and bladder cancer is well established. Thus, tumor-specific mutations in plasma of carcinogen-exposed individuals were associated with elevated risk of cancer [42, 43], and the frequency of *TP53* mutations at codon 248, 249, and 273 in plasma of smokers was associated with years of smoking history [43]. Gormally et al. found that *KRAS2* and *TP53* mutations in plasma of healthy individuals were associated with increased risk of developing bladder cancer [42]. Moreover, *TP53* mutations in exons 5, 6, 7, or 8 are associated with disease recurrence. Microsatellite abnormalities and *TP53* mutations predicted early disease recurrence. Additionally, increased plasma ccfDNA indicates possible systemic disease at diagnosis [44]. Massively parallel sequencing at high depth, as well as other novel technologies for mutation detection such as TAm-Seq, should enable future clinical translation of ctDNA detection in LnCa patients.

3.4.3.2 Circulating Microsatellite Alterations as LnCa Biomarkers

The clinical relevance of detecting circulating MSA (LOH and MSI) in LnCa has been addressed. Ahrendt et al. detected MSI in bronchial cells from patients with early stage localized small tumors, indicative of their presence early in lung carcinogenesis [45]. Sozzi et al. had pursued MSA for early diagnosis of NSCLC. LOH and MSI were analyzed with two markers (D21S1245 for MSI and LOH at the *FHIT* locus) that are very unstable in LnCa. MSAs were present in 56 % of tumor samples and of this, plasma samples were positive for 61 % of cases. Early detection implication was revealed by the fact that 43 % of the positive cases were stage I disease patients, and 45 % had maximum tumor diameter of 2 cm [46]. Allelic loss at chromosome 3p occurs early in the development of LnCa, being

observed in 78 % of preneoplastic bronchial lesions and in >90 % of NSCLC. MSA at this locus examined with four markers (D3S1300, D3S1289, D3S1266, and D3S2338) were significantly associated with lung SCC [44].

Chen et al. examined MSI, which is present in up to 50 % of SCLC patients and was detectable in 76 % of tumor samples and 71 % of matched plasma samples [47]. LOH in plasma of SCLC patients is mostly found in those with advanced stage invasive cancers, suggestive of metastatic nucleic acids in plasma. Both tumor and plasma samples of SCLC patients were assayed in a prospective study. MSI analysis included the following polymorphic markers, ACTBP2, UT762, and AR, that are frequently altered in SCLC. With all these markers, the detection rate of alterations in plasma (i.e., at least one in plasma sample) was 71 %. The positive presence of MSI and *TP53* mutations (both at the same time) conferred poor survival [48]. Twelve microsatellite markers covering nine chromosomal regions in both SCLC and NSCLC patient plasma samples were studied, and allelic imbalance (AI) was observed in as many as 83 % of patients. Of paired tissue–plasma samples, 83 % had tissue AI of which 85 % was in plasma DNA. Control plasma and bronchial DNA were all negative for MSA [49].

Ludovini et al. prospectively studied three biomarkers (MSA, *TP53* mutations, and plasma DNA concentration) for their clinical utility in LnCa patients [44]. MSA was studied with three markers on chromosome 3p and *TP53* mutations in exons 5, 6, 7, and 8. Mean plasma DNA concentration was significantly higher in cancer patients and was much lower in responders and decreased progressively. MSA was in 39.5 % and *TP53* mutations in 54 % of tumor samples. MSA were associated with squamous cell NSCLC, *TP53* mutations with lymph node metastasis, and both were associated with disease recurrence.

From all these studies, the consensus is that these biomarkers are qualitatively detectable and quantitatively measurable in plasma from LnCa patients, but rarely in plasma from healthy individuals. Some tumor genetic signatures (e.g., MSA) are heterogeneous and differ between tumor and plasma of the same patients. This is partly accounted for by the heterogeneous cell populations of tumors, especially because migration requires acquisition of additional genetic changes. Quantitative analysis using various methodologies reveals different concentrations of mutations in plasma of patients and controls. However, all studies indicate significantly higher concentration of plasma nucleic acids in patients compared to controls. Additionally, there is a general trend of decreasing plasma DNA concentrations in patients responding to treatments on follow-up, making this noninvasive serial measurement useful in monitoring complete surgical resection, as well as chemotherapy and radiotherapy efficacy.

3.4.4 Circulating LnCa Coding RNA Biomarkers

While probably not the most attractive circulating biomarker, the transcripts of a number of genes appear clinically promising as LnCa biomarkers. Human *TERT*

and *EGFR* mRNA were assayed in sera from LnCa patients. Multivariate analysis revealed that *hTERT* transcript copy number independently correlated with tumor size, multiplicity, metastasis, recurrence, and smoking status. Similarly, *EGFR* mRNA correlated with clinical stage of tumors. For LnCa detection, circulating *hTERT* and *EGFR* transcripts performed at sensitivities of 89 % and 71.3 % and specificities of 72.7 % and 80 %, respectively. Serum levels significantly correlated with tissue levels, and a significant decrease in serum *hTERT* mRNA occurred after surgery [50].

A panel of five gene transcripts was examined in plasma from LnCa patients. While three of these targets (*KRT19*, *MAGE-2*, and *TIF-1*) were of no diagnostic value, *hnRNP* and *HER2/Neu* were able to detect 78 % and 39 % of cancers, respectively. However, combining both genes enabled identification of all tumors [51]. Expression of *hnRNPB1* mRNA in plasma from patients with early stage LnCa including those with dysplastic lesions, benign lung diseases, and healthy controls uncovered significantly much higher mean transcript levels in cancer patients than controls. In agreement with their previous detection by immunohistochemistry, SCC patients had higher levels than patients with adenocarcinoma [52]. The usefulness of plasma *hnRNAB1* mRNA in differentiating NSCLC from benign lung diseases, especially tuberculosis has been investigated. *HnRNA* mRNA could be measured in plasma from 93.3 % of NSCLC patients, and the levels were significantly much higher (normalized mean level 62.2) than in patients with benign lung diseases (normalized mean level 2.7) [53].

Circulating levels of *5T4* (a trophoblast glycoprotein overexpressed in epithelial cancers) mRNA in sera from patients with breast and LnCa revealed a detection rate of 42 % in cancer patients compared with 12 % of controls [54]. Other gene transcripts assayed in LnCa patient sera include *EGFR* and *lung X*. *EGFR* mRNA as a measure of CTCs was detected in circulation of 16 % of patients with lung, colon, and pancreatic cancers, but not in healthy controls. *Lung X* (lung-specific X protein) is a gene Iwao et al. first identified and later demonstrated to be expressed in 58 % of blood samples from NSCLC patients [55, 56].

3.4.5 Circulating LnCa Noncoding RNA Biomarkers

Circulating miRNA has been well studied in LnCa, consistent with the interests of several groups in trying to identify ccfDNA and other biomarkers that can be sampled noninvasively for the management of this particular cancer (Table 3.3). Obviously, miRNA is attractive for reasons of specificity and stability in circulation.

Table 3.3 Circulating miRNA altered in LnCa Patients

Diagnostic biomarkers		Prognostic biomarkers	
Increased	Decreased	Increased	Decreased
let-7a, miR-17, miR-17-3p, miR-17-5p, miR-19b, miR-21, miR-25, miR-27a, miR-28-3p, miR-29c, miR-30c, miR-30d, miR-92a, miR-106a, miR-140-5p, miR-146b, miR-155, miR-182, miR-191, miR-192, miR-197, miR-203, miR-205, miR-210, miR-212, miR-214, miR-221, miR-223, miR-451, miR-499, miR-660, miR-1254	miR-16, miR-17-5p, miR-24, miR-29c, miR-126, miR-141, miR-145, miR-200, miR-452*, miR-486-5p, miR-518a-5p, miR-574-5p, miR-593*, miR-663, miR-718, miR-1228*, miR-1972, miR-2114	miR-21, miR-28-3p, miR-30d, miR-106a, miR-140-5p, miR-155, miR-197, miR-486, miR-486-5p	miR-1, miR-15b, miR-16, miR-17, miR-126, miR-142-3p, miR-148a, miR-221, miR-499

Diagnostic biomarkers: increases or decreases are discriminatory between cancer and healthy controls. Prognostic biomarkers: decreases or increases correlate with survival outcomes or aggressive and metastatic disease

3.4.5.1 Circulating miRNA as LnCa Diagnostic Biomarkers

Analysis of twelve exosomal miRNAs in plasma revealed elevated expression in LnCa patients compared to controls [57]. Indeed, exosomal miRNA concentration is much higher in patients than controls. An approach involving Solexa sequencing of global serum miRNA identified 63 circulating miRNAs in samples from NSCLC patients that were not present in healthy controls. However, only miR-25 and miR-223 were identified as the best diagnostic biomarkers of NSCLC. Other circulating miRNAs that are of possible diagnostic use in NSCLC include miR-21, miR-126, miR-145, miR-155, miR-210, and miR-486-5p [58]. Foss et al. identified miR-574-5p and miR-1254 as diagnostic biomarkers of LnCa [59]. Bianchi et al. [60] and Boeri et al. [61] explored the possibility of identifying circulating miRNAs that could be used for screening of asymptomatic population. Bianchi's group identified 34-miRNA signatures that discriminated NSCLC from benign lesions, and miR-28-3p, miR-30, miR-92a, miR-140-5p, and miR-660 appear to have potential for early detection of LnCa in asymptomatic individuals. The samples for this study were collected over a year prior to clinical diagnosis and were still accurate in detecting LnCa. Boeri's series uncovered miR-17, miR-19b, miR-92a, miR-106a, and miR-660 as informative early diagnostic biomarkers. Other potential early detection circulating LnCa miRNAs are miR-16, miR-452*, miR-518a-5p, miR-574-5p, miR-593*, miR-663, miR-718, miR-1228*, miR-1972, and miR-2114 [62].

Shen et al. performed a meta-analysis of circulating miRNA for the diagnosis LnCa. The 18 studies (spanning 2011–2012) included in this analysis comprised of 1187 patients and 879 controls. Although the studies were heterogeneous, the diagnostic performance achieved a pooled sensitivity of 85 %, specificity of 84 %, PLR of 5.23, NLR of 0.20, DOR of 31.77, and SAUROCC of 0.92, indicative of their diagnostic potential [63].

3.4.5.2 Circulating miRNA as LnCa Prognostic Biomarkers

A number of studies have explored miRNAs in outcome predictions using pre-diagnosis and pretreatment plasma and serum samples. Genome-wide sequencing of serum samples uncovered miR-1, miR-30d, miR-486, and miR-499 as predictive of NSCLC patient survival [64]. This miRNA signature was an independent predictor of OS. MiR-21 is associated with lymph node metastasis, advanced stage disease, patient survival, treatment prediction as to sensitivity to platinum-based chemotherapy, and is useful for disease monitoring after surgery [65–68]. A 3-year survival in patients with high circulating levels of miR-21 is 39.8 % compared with 58.2 % for patients with low levels. High levels of miR-21 are associated with worse outcome in several other malignancies and are an independent prognostic factor for NSCLC, with pooled HR of 2.153 [69]. Circulating miR-125b is identified as a screening biomarker that also predicts worse outcome in NSCLC patients. In a meta-analysis, high miR-125b levels were associated with poor survival of NSCLC patients with pooled HR of 2.33 [70]. MiR-142-3p predicts LnCa recurrence [71], and miR-155, miR-182, and miR-197 levels are lower in patients who respond to chemotherapy [72].

3.4.6 Circulating LnCa Serum Protein Biomarkers

Protein biomarkers for LnCa include traditional serum proteins and novel identified and unidentified proteins and peptides from proteomic studies. A number of conventional or traditional serum markers with proven clinical utility are recommended for LnCa diagnosis, prognosis, and monitoring for treatment response and recurrence. These include CYFRA 21-1, CEA, NSE, and ProGRP (Table 3.4). Of the four serum biomarkers, the first two have been most extensively studied.

3.4.6.1 Serum CYFRA 21-1 as LnCa Biomarker

Fragments of cytokeratin in circulation are investigated for clinical utility in LnCa. Among them are cytokeratin 19 fragments (CYFRA 21-1); cytokeratin 18 fragments or tissue polypeptide-specific antigen (TPS); cytokeratin 8, 18, and 19 fragments or

Table 3.4 Serum biomarkers recommended by the NACB and/or EGTM for management of LnCa

Biomarker	Diagnostic	Prognosis	Treatment monitoring	Recurrence monitoring
CYFRA 21-1	NSCLC	NSCLC	NSCLC/SCLC	NSCLC/SCLC
CEA	NSCLC	NR	NSCLC (adenocarcinoma)	NSCLC (adenocarcinoma)
NSE	SCLC	NR	SCLC	SCLC
ProGRP	SCLC	NR	SCLC	SCLC

NR not recommended

tissue polypeptide antigen (TPA); and M30. CYFRA 21-1 is the most sensitive and specific for NSCLC diagnosis, especially SCC subtype. However, the levels of CYFRA 21-1 are elevated in SCLC as well as numerous other cancers (breast, gastrointestinal tract, head and neck, and bladder cancers). Elevated levels of CYFRA 21-1 over 3.3 ng/ml in serum are associated with NSCLC, especially those with squamous cell histology, with a diagnostic sensitivity and specificity of up to 59 % and 94 %, respectively.

Serum CYFRA 21-1 as LnCa Prognostic Biomarker

Traditionally, the TNM staging system is clinically used for prognostication of solid tumors. For LnCa, other parameters including weight loss and performance score are included in prognostic predictions, and hence treatment decision-making. Numerous studies have proven the prognostic value of CYFRA 21-1 in NSCLC. A retrospective large study indicated CYFRA 21-1, NSE, performance status, and tumor stage were independent prognostic biomarkers for NSCLC. A meta-analysis involving 11 studies with a total of 2003 NSCLC patients concluded that CYFRA 21-1 was a strong independent prognostic biomarker for NSCLC patients as a whole and in a subset of patients with early and late stage tumors. The National Cancer Advisory Board (NCAB) and European Group on Tumor Markers (EGTM) have recommended it for such uses (Table 3.4). Because evidence favor complementary role of biomarkers in LnCa prognosis, it has been suggested that biomarkers should be included in the TNM staging system. Thus, a TNMB (B for biomarkers) model should be adopted for LnCa prognostication [73].

Serum CYFRA 21-1 as Postoperative Monitoring LnCa Biomarker

CYFRA 21-1 is also recommended for the postoperative surveillance of LnCa patients. Surgical maneuvers usually result in biomarker release from tumor and normal cells into the circulation. In NSCLC, following curative-intent tumor removal, the levels of CYFRA 21-1 (and also TPA, SCCA) with a short half-life of between 1.5 and 3 h should fall in a couple of days. Failure to decline within reference range (<3.3 ng/ml) indicates the presence of residual or micrometastatic disease. This raises concerns of possible early recurrence and the need for adjuvant chemotherapy.

Serum CYFRA 21-1 as Systemic Therapy Response Monitoring Biomarker in Advanced Stage LnCa

Tumor death following chemotherapy or radiotherapy causes release and hence an acute rise in circulating biomarkers. But these high levels of serum markers decline sharply. Declining levels to reference range (of healthy individuals) indicate response, while failure to decline, or even worse, rising biomarker levels indicate progressive disease. In advanced stage LnCa patients on systemic chemotherapy or radiotherapy, response is monitored clinically with conventional imaging (CT scans) performed 2–3 months following treatment. Using the Response Evaluation Criteria In Solid Tumors (RECIST) guidelines, complete response is when any target lesion is undetectable, while partial response means 30 % reduction in tumor size. However, for progressive disease, an increase in tumor size ≥20 % should be demonstrated. For tumors that fail to reduce by 30 % or increase more than 20 %, the designation of stable disease is applied. While this assessment is useful clinically, biomarkers in circulation or body fluids are emerging as having more powerful utility in such clinical scenarios, not only because of their noninvasive and ease of serial sampling, but they tend to decline faster, with lead times of several months compared to imaging. Circulating biomarkers in response monitoring are measured in relation to the gold standard (imaging), and for LnCa, the evidence for CYFRA 21-1 has been consistently positive.

Circulating biomarker levels have been investigated for two main possible uses to monitor and guide treatment decision-making: first, its value after third cycle of treatment and, second, after first cycle of systemic therapy, such that modifications can be made to earlier treatment decisions and thus spare the persistent toxicity offered to nonresponders. To this end, marker level kinetics has been proposed as a means to monitor tumor dynamics during therapy. Performances based on defined kinetic parameters have varied but show a consistent trend. Van de Gaast et al. used a 65 % reduction in CYFRA 21-1 as indicative of response, while an increase of up to 40 % was a measure of disease progression [74]. With these kinetic parameters, clinical tumor and biochemical responses were concordant at 74 %. Using this same set of parameters, Ebert et al. found the biochemical and clinical response rates to be concordant at 59 % [75]. In this series, the concordant rate was the same even when they set the levels of CYFRA 21-1 reduction at 30 % as response and increase of 30 % as treatment failure. CYFRA 21-1 levels increased after first-line chemotherapy as expected. However, following third cycle treatment, 88 % of patients with reductions >70 % (from baseline elevated levels) or those who returned to within normal levels were clinically responsive. On the contrary, increased levels over 10 % were associated with progressive disease as was evident in 60 % of such patients [76]. Similar performances have been reported in patients with inoperable NSCLC where decreases <35 % indicate response to treatment.

The need to know and hence modify treatment early is useful not only from an economic perspective but from the central Hippocratic dogma of medicine, “first do no harm” to the patient. Thus, studies have explored the possible value of serum biomarkers for response assessment especially after first-line systemic therapy. Holdenrieder et al. [14, 16, 17, 77] provide evidence for such utility of serum

biomarkers in LnCa. In a study involving 212 prospective patients after first cycle systemic therapy, serum CYFRA 21-1, nucleosomal levels, stage, and therapy before second cycle were independent predictors of response in multivariate analysis [16]. In an expanded patient cohort (311), 29 % of those with progressive disease (i.e., nonresponsive to systemic therapy) were predicted by a combination of CYFRA 21-1 and nucleosomal DNA levels at a specificity of 100 % [17]. A prospective analysis of 161 patients further confirmed these findings of treatment response. Circulating nucleosomes, CYFRA 21-1, NSE, ProGRP, and CEA were measured at diagnosis and before each cycle of chemotherapy in patients with SCLC. Remission was observed in 81 %, stable disease in 6 %, and disease progression in 13 %. Comparative analysis of marker levels after first-line treatment revealed that, with the exception of CEA, biomarker levels were significantly lower in responders than those with progressive disease [77]. In another study of patients with recurrent NSCLC, the above biomarkers were measured during cycles of treatment. Consistent with their earlier findings, decreasing CYFRA 21-1, and nucleosome levels after first and second cycle treatments corresponded to response to treatment. ProGRP was uninformative in this study, consistent with its strong association with SCLC [14, 77].

Serum CYFRA 21-1 as LnCa Recurrence Monitoring Biomarker
Similar to treatment response, recurrence or relapse is very sensitive to circulating biomarker dynamics, demonstrating detective ability several months of lead time compared to conventional imaging. CYFRA 21-1 levels decreased to normal after tumor resection but increased in 78 % of patients with recurrences. This was 2–15 months before the gold standard (imaging) could detect these recurrences in some cases. When considering only patients with preoperative elevated CYFRA 21-1 (i.e., levels >3.3 ng/ml), the assay was 100 % sensitive and specific at detecting relapses [78].

3.4.6.2 Circulating CEA, NSE, and ProGRP as LnCa Biomarkers

Carcinoembryonic antigen (CEA) is an oncofetal protein secreted by many tumors. Their levels are elevated in ~40 % of NSCLC patients, especially those with the adenocarcinoma subtype. High levels are predictive of potential brain metastasis and poor prognosis. As well, smoking elevates the levels. Neuron specific enolase (NSE) or γ-enolase is a glycolytic enzyme associated with tissues of neuroectodermal origin. Other cell types including lymphocytes, platelets, and renal epithelial cells also express it. Because SCLC is of neuroendocrine origin, levels of NSE are significantly elevated in sera from these patients compared to patients with NSCLC and other tumors. Levels >25 ng/ml were detected in 72 % of SCLC patients compared to only 8 % of patients with other lung tumors. The elevated levels were more frequent in patients with advanced stage disease (91 %) compared to those with limited disease (50 %). ProGR is a precursor of gastrin-releasing peptide and hence is associated with tissues of the gastrointestinal tract,

but also lungs and the nervous system. Levels of ProGRP are elevated in sera from the majority of SCLC (73 %) compared to NSCLC patients.

3.4.7 Circulating LnCa Proteomic Biomarkers

Proteomic approaches have been employed in biomarker discovery for LnCa. Both protein peak or *m/z* signatures and identified peptides and proteins show differential levels between LnCa patients and controls.

3.4.7.1 Circulating Peptide Spectral Peak Signature LnCa Biomarkers

Several proteomic analyses of serum samples have used protein peaks or spectral data to discriminate between LnCa patients and healthy controls. Five peaks with *m/z* of 2538, 5335, 6429, 8245, and 11,493 Da achieved a sensitivity of 86.9 % (as high as 79 % for even early stage I/II disease) and a specificity of 80 %. This protein peak signature was more sensitive in patients with NSCLC (where sensitivity reached 91.4 %) than those with other LnCas [79]. Using support vector machine, SELDI-TOF MS spectral data could distinguish SCLC patients from those with pneumonia, as well as patients with NSCLC from healthy individuals at a sensitivity of between 83–88 % and specificity of 75–91 %. These diagnostic protein peaks were superior to NSE in performance [80]. Sreseli et al. identified 17 MS protein signature that was used in two validation studies [81]. These peaks could separate LnCa from healthy individuals at a sensitivity and specificity of 87.3 % and 81.9 %, respectively, in the first validation and 90 % and 67 % in the second validation cohorts. In another study, serum peptides were captured and concentrated using magnetic bead-based weak cation exchange on the ClinProt robotic platform. Analysis with MALDI-TOF MS identified five protein patterns that could differentiate SCLC patients from healthy controls at a sensitivity and specificity of 90 % and 97 %, respectively. This serum protein fingerprint could detect 89 % of stage I/II SCLCs.

3.4.7.2 Identified Circulating LnCa Proteomic Biomarkers

A number of serum peptides, proteins, and glycoproteins have been identified that discriminate LnCa patients from healthy controls. One protein consistently assayed by several investigators as being elevated in LnCa patient samples is an 11.6 kDa protein, serum amyloid protein A (SAA). Serum amyloid protein A is an apolipoprotein associated with HDL. These acute phase reactants consist of three isoforms, SAA1, SAA2, and SAA3. The first two are expressed in inflammatory liver disease, while expression of SAA3 is induced in distinct tissues and organs. The biologic relevance of SAA expression by LnCa cells was demonstrated in an in vitro

experiment. LnCa cell lines secrete SAA1 and SAA2 that stimulate macrophages to induce expression of matrix metalloproteinase 9 (MMP9) involved in the metastatic cascade [82].

Howard et al. identified serum amyloid protein with *m*/*z* of 11,702 as LnCa biomarker, and it was validated by ELISA [83]. Other workers have identified many proteomic serum biomarkers of LnCa, many of which include SAA. An 11.6 kDa protein later identified as SAA had a sensitivity of 84 % and specificity of 80 % in discriminating LnCa from controls [84]. MALDI-TOF MS performed on unfractionated training and test serum sample sets uncovered seven proteomic features that could detect LnCa at an overall accuracy of 78 % for the training set and 72.6 % for the test set. In this study, the diagnostic peptides of *m*/*z* 11,500 Da were identified as a cluster of truncated forms of SAA [85]. Of 49 differentially expressed proteins, 4 that showed high spectral counts were identified as SAA, alpha-1 acidic glycoprotein 1 and 2 (AAG1, AAG2), and clusterin. Of five spectral peaks (11,530, 11,700, 13,780, 13,900, and 14,070) differentially expressed in LnCa, sequence identification revealed SAA and transthyretin and its variants as LnCa biomarkers [86]. SAA1 and SAA2 levels were markedly elevated in sera from LnCa patients compared to controls by mass spectrometry. These findings were verified by Western blot and ELISA quantification. In NSCLC and SCLC tissue samples, SAA levels were equally elevated by IHC. Using ELISA quantification with 50 ug/ml as a diagnostic cutoff, a sensitivity of between 50 and 70 %, and specificity of 95 % were achieved [82]. Isotype-specific proteomic quantification (multiple reaction monitoring, MRM) was used in a subsequent study to confirm the elevated expression of SAA in sera from LnCa patients [87]. There is evidence that serum SAA levels may have prognostic value as well. The work by Cho et al. revealed that elevated SAA was associated with dismal outcomes [88]. However, levels of SAA are elevated in cancers of the pancreas, colorectum, kidneys, and ovaries; hence, the prognostic utility may be more informative than its diagnostic applications.

Other proteins identified by MS analysis of serum samples from LnCa patients include haptoglobin α-subunit, hepatocyte growth factor, transthyretin, apolipoprotein A4, fibrinogen α-chain, and limbin. Haptoglobin α-subunit and hepatocyte growth factor were associated with SCLC, while peptides from apolipoprotein A4, fibrinogen α-chain, and limbin were potential early LnCa biomarkers [89]. Native transthyretin (*m*/*z* 13,780, 13,900, and 14,070) levels were lower in LnCa patients compared to controls, with a diagnostic sensitivity and specificity of between 65 and 75 % [90]. Thirty-eight glycoproteins could discriminate NSCLC from controls. Three of the proteins were validated using ELISA, and their levels were consistent with the MS data. Alpha 1-antichymotrypsin, insulin-like growth factor-binding protein 3, and lipocalin-type prostaglandin D synthase were among these glycoproteins [91].

3.4.8 Circulating LnCa Cells

Various methods have been employed to enrich, isolate, detect, and characterize circulating LnCa cells (CLnCaCs) in patients with primary LnCa. Both prognostication and treatment response predictions are important in oncology, and the clinical potential of CLnCaCs in prognosis and treatment monitoring has proven useful.

3.4.8.1 CLnCaCs as Prognostic and Treatment Response Biomarkers in SCLC

Cristofanilli et al. established a prognostic threshold of 5 CTCs per 7.5 ml of blood for the CELLSEARCH® system in patients with breast cancer, and this standard threshold has been used in many other studies including LnCa. In advanced stage SCLC, CLnCaC parameters have been correlated with disease outcome. CLnCaCs have been detected at a high frequency of 86 % in SCLC patients. Similarly, the cell counts were high in this cohort, with median CLnCaCs per 7.5 ml of blood being 28 (range 0–44,896). Adverse prognostic outcome was associated with high CLnCaC counts. Thus, patients with CLnCaCs > 300 had a 4.5 months median survival compared to 14.8 months in patients with counts of <2. The lack of CLnCaC reduction during chemotherapy strongly conferred poor prognosis [92]. Another prognostic study by this group involved enumeration of CLnCaCs and circulating tumor microemboli (CTM) in SCLC patients on chemotherapy [93]. Baseline CLnCaCs was positive in 85 % of patients before therapy, of which 32 % had CTM. Baseline CLnCaCs were significantly associated with PFS and OS ($p < 0.0001$), and this finding remained an independent prognostic factor in multivariate analysis. The absence of CLnCaC decline to <50 cells after treatment was predictive of worse outcome. Naito et al. detected CLnCaCs (≥ 2) in 68.6 % and 26.5 % of SCLC patients at baseline and following chemoradiation, respectively, but the detection frequency subsequently increased to 67.6 % due to relapses [94]. CLnCaCs > 8 was significantly associated with poor OS, and patients in which CLnCaCs were still detectable after treatment and those in relapse had worse outcomes.

A multicenter study enumerated CLnCaCs in SCLC patients before, after one cycle, and at the end of chemotherapy. Patients with localized disease had lower CLnCaC counts (median CLnCaCs $= 6$) than those with metastatic disease (median CLnCaCs $= 63$). The absence of CLnCaCs was associated with good clinical outcome. Decreases in CLnCaC counts after first cycle chemotherapy predicted better OS ($p = 0.004$). Importantly, CLnCaC enumeration was more superior to conventional prognostic features such as disease stage and CT scans. Normanno et al. similarly enumerated CLnCaCs at baseline and after first cycle of chemotherapy in SCLC patients [95]. Probably due to the advanced stage disease among this cohort, CLnCaCs were detected in as many as 90 % of the patients, and the counts

were associated with the number of organs involved. A better outcome was predicted by an over 89 % reduction in CLnCaCs after therapy.

Igawa et al. questioned the value of CLnCaC detection by the OBP-401 assay (TelomeScan®) and its prognostic importance in SCLC patients [96]. This method enables capture of viable cells, by incubation of blood with telomerase-specific, replication-selective, oncolytic adenoviral agent carrying a GFP. Viable CLnCaCs were assayed before and after treatment and were positive in as many as 96 % in this cohort. Patients with baseline CLnCaCs < 2 per 7.5 ml of blood survived longer than those with two or more CLnCaCs (14.8 months vs. 3.9 months, $p = 0.007$). Baseline CLnCaC count was an independent prognostic variable of survival in multivariate analysis. Moreover, PFS after two cycles of therapy was longer for patients with <2 CLnCaCs than those with more than 2.

3.4.8.2 CLnCaCs as Prognostic and Treatment Response Biomarkers in NSCLC

The negative selection MAINTRAC technique was used to analyze postoperative blood samples from NSCLC patients. Of 30 patients with resectable NSCLC, 86 % had positive CLnCaC counts preoperatively, but following surgery, all were positive for CLnCaCs. Increasing CLnCaCs after surgery were associated with elevated risk of relapse or recurrence [97]. In preoperative blood of patients with resectable NSCLC, non-immunogenic CLnCaC capture was undertaken and correlated with prognosis. Forty-nine percent of the 208 patients were positive for CLnCaCs, defined by the authors as circulating non-hematological cells (CNHCs). Patients with CNHCs of $\geq$50 had a shorter DFS and OS [98]. CLnCaCs were enumerated in advanced stage III/IV NSCLC patients prior to and after one cycle of chemotherapy. Expectedly, the numbers of CLnCaCs correlated with disease stage, such that CLnCaC counts of $\geq$2 were present in none of stage IIIA, but positive in 7 % of stage IIIB and in as many as 32 % of stage IV disease patients. CLnCaC counts were equally more in stage IV (mean = 60) than in stage III (mean = 27) disease patients. CLnCaCs $\geq$ 5 per 7.5 ml of blood conferred significant poor PFS and OS in both univariate and multivariate analyses. A better prognosis was also observed in patients with decreasing CLnCaCs after chemotherapy compared to those with increasing counts [99]. Hirose et al. performed a prospective enumeration of CLnCaCs in patients with metastatic NSCLC in relation to treatment [100]. CLnCaCs failed to predict treatment response; however, CLnCaC-positivity was associated with worse PFS. CLnCaCs enumerated before first, second, and fifth cycle of treatment of NSCLC patients were predictive of survival. Intact CLnCaCs were isolated in 41.9 % of these patients, and patients with $\geq$5 CLnCaCs had shorter PFS and OS than those with $<$5 cells. A decrease in CLnCaCs after second treatment regimen was associated with improved PFS and OS, suggestive of response to therapy [101]. Maheswaran et al. used a CTC chip to analyze blood samples from 27 patients with advanced stage NSCLC [102]. Twenty of them had known *EGFR* mutation status. Median CLnCaCs per ml of blood was 74, and all

27 patients were positive (100 % detection rate). Sequence analysis of *EGFR* mutation was performed on CLnCaCs, and this was concordant in 95 % of those with known *EGFR* mutation status. The *EGFR* T790M mutation was detected in 64 % of nonresponders compared to 33 % of responders to anti-EGFR TKI treatment. CLnCaC enumeration indicated a decline with imaging assessment of response, but increased in patients with disease progression.

In both NSCLC and SCLC patients at various diagnostic stages, CLnCaC counts correlated with the extent or stage of tumors. CLnCaC detection in this cohort was low (30.6 %) compared to other studies that used the same CELLSEARCH® system. However, given that the majority of patients (110/150) were early stage disease (stage I/IIIA), this finding seems reasonable. While this study was not too great at discriminating between cancer and controls (12 healthy controls were positive), the AUROCC was 0.783 ($p < 0001$) in predicting distant metastasis, and CLnCaCs were good at discriminating stage I from stage IV LnCa [103]. Advanced stage NSCLC patients on treatment were investigated for treatment response based on CLnCaCs. Baseline CLnCaCs was positive in 78 % of these patients. There was a significant correlation between increasing CLnCaCs and worse response to treatment ($p = 0.009$), and decreasing CLnCaCs was associated with better PFS ($p = 0.05$) [104].

3.4.8.3 Other Applications of CLnCaCs

Circulating cancer cells inform tumor biology, evolution, and hence optimal patient management. Thus, some investigators have evaluated the usefulness of CTCs in cancer-specific mutation detection, the ability to sequence the genome of a single CLnCaC, and the clinical importance of serial CLnCaC sampling. These are therefore mainly pilot feasibility studies, but with great clinical implications.

In NSCLC patients refractory to EGFR TKI therapy, CLnCaCs and *EGFR* mutations were detected at frequencies of 33.3 % and 25 %, respectively [105]. Mutations in ccfDNA was significantly higher in patients with >2 CLnCaCs than those with less. These findings suggest *EGFR* therapy-resistant mutation detection in ccfDNA correlate with aggressive tumors that have high CLnCaCs. However, whether these mutations are from lysed CLnCaCs in circulation is unclear. Marchetti et al. undertook a prospective multicenter study of patients enrolled in the TRIGGER study of advanced stage NSCLC who were receiving erlotinib treatment because of activating *EGFR* mutations in their tumors. CLnCaCs, ranging from 1 to 29, were detected in 41 % of the patients [106]. Using next-generation sequencing (NGS), *EGFR* mutations (mostly exon 19 deletions and exon 21 mutations) were detected in 84 % of CLnCaC preparations from the patients, and these corresponded to those in matched tumor tissues. NGS thus offers the possibility of real-time mutation analysis in serial CLnCaCs. Another proof of principle study explored sequencing of single CLnCaC exomes to characterize mutations. CLnCaCs were captured followed by FACS and whole-genome amplification prior to sequencing. This method enabled 55 % of the exome

to be sequenced at 20-times depth, enabling 72 % genome variation calls to me made [107].

The biology of ex vivo CLnCaC manipulation was assessed. CLnCaCs from patients with SCLC who were either chemosensitive or chemoresistant were noticed to be the same as CLnCaC-derived explants with respect to therapy [108]. The biologic behavior of these ex vivo CLnCaCs was similar to those in the patients, suggesting that serial molecular analysis of CLnCaCs for tumor behavior, which is clinically relevant, can be achieved.

The dynamics of CLnCaCs during surgery has been examined. CLnCaCs were evaluated in pulmonary venous blood samples obtained before and after lobectomy [109]. Prior to surgery, CLnCaCs were detected in pulmonary venous blood at a frequency of 73 %, but only 6.7 % of peripheral blood samples were CLnCaC-positive. CLnCaC numbers increased significantly following surgery (median values were 4.0 CLnCaCs per 2.5 ml of blood before vs. 60.0 CLnCaCs/ml of blood after surgery). This increase in CLnCaCs after surgery appears to be associated with microlymphatic tumor invasion. A 5-year follow-up clinical data is anticipated.

Because many studies of CLnCaCs had relied on the CELLSEARCH® method, it was compared to the membrane filtration system ISET (rare cells SA) for CLnCaCs isolation in stage III/IV NSCLC patients. Same samples were processed using both techniques, and the ISET technique appeared more superior to CELLSEARCH® by detecting CLnCaCs of ≥2 cells in 77 % of patients compared to 23 % by the CELLSEARCH® system. Numerical CLnCaCs were also higher by ISET than the CELLSEARCH® system (median CLnCaCs were 127 per 7.5 ml of blood by ISET compared to 10 per 7.5 using CELLSEARCH® system) [110]. Whether these findings are generalizable to their performances in other solid tumors is unclear.

3.5 LnCa Extracellular Vesicles

The biologic importance of extracellular vesicles (EV) in cancer progression is well established. An important cargo of EVs used in cancer cell horizontal communication is miRNA. While not the focus of many miRNA studies, two studies have revealed that LnCa-derived EVs are packaged with miRNAs. Rabinowits et al. demonstrated that 12 miRNAs that are elevated in NSCLC tissues compared to normal lung tissue were selectively packaged in cancer-derived exosomes [57]. The role of these miRNAs in communications between NSCLC cells and other cells need further elucidation. Another study revealed the prognostic role of LnCa-derived circulating extravesicular miRNA. Silva et al. showed that plasma EVs from NSCLC patients contain let-7f and miR-30e-3p with significant prognostic prediction [111].

3.6 Commercial Circulating LnCa Biomarker Products

Resolution Bio has developed a ctDNA assay for LnCa (Resolution Bio ctDx™ Lung), based on its proprietary platform. The Resolution Bio ctDx™ platform enables analysis of multiple patient samples on a single desktop sequencer. The workflow involves the use of proprietary biochemistry to amplify ccfDNA, followed by target capture of ctDNA using very small probes for genetic analysis. The system detects SNPs, insertions/deletions, copy number variations/amplifications, and gene fusions/translocations. Importantly, prior knowledge is dispensable for fusion gene identification. The ctDx™ Lung assay includes detection of SNPs in *ALK*, *BRAF*, *EGFR*, *HER2*, *KRAS*, *NRAS*, *MEK1*, *MET*, *PIK3CA*, *RET*, and *ROS1*; fusions in *ALK*, *NTRK1*, *RET*, and *ROS1;* copy number variations in *EGFR*, *HER2*, and *MET*; and alterations in *KEAP1*, *STK11*, and *TP53* tumor suppressor genes. This is one clinical application of liquid biopsy as it informs clinical decision-making on patient management.

3.7 Summary

- LnCa remains one of the major cancers in the world in terms of incidence and mortality, despite the extensive accumulated molecular genetic information on this disease.
- Early detection of LnCa continues to be problematic, resulting in dismal 5-year survival outcomes.
- The molecular pathologic information on LnCa is ushering in a large number of targeted therapies.
- There are multitudes of molecular biomarkers in body fluids of LnCa patients.
- The development of noninvasive early detection products that inform treatment decisions based on genetic profiles of tumors should help with improved patient survival.
- Because LnCa demonstrates field cancerization, the development of field-cancerized molecular biomarkers should enable risk identification for primary prevention, as well as biomarkers for profiling tumor margins to identify patients at risk for developing second primary tumors after surgery. Such patients can be offered adjuvant therapy.
- The clinical potential utility of CLnCaCs is encouraging and deserves actionable effective product development.
- Noninvasive LnCa tests based on ctDNA are highly commendable.

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Chapter 4
Breast Cancer Biomarkers in Circulation

Key Topics

- Brast cancer (BrCa) screening
- Molecular pathology of BrCa
- Circulating BrCa biomarkers
- Circulating BrCa cells and stem cells

Key Points

- BrCa is a disease that has benefited from recent molecular genetic research, because of the well-characterized molecular pathology. For instance, despite the numerous histopathologic subtypes, molecular profiling recognizes only four relevant subgroups with prognostic and predictive potential. Gene expression profiling has uncovered clinically actionable sets of genes used in the Oncotype DX® and MammaPrint BrCa tests.
- Noninvasive tests for BrCa hold potential for advancing acceptable disease detection and management. The detection and quantification of ccfDNA, epigenetic alterations (e.g., *CDKN2A* methylation), mutations (e.g., *TP53*), microsatellite alterations (e.g., D13S2118 alterations), mRNA (e.g., *hTERT*), ncRNA (e.g., miR-155), proteins, and metabolites (e.g., choline-containing compounds) in circulation should add to the arsenal of biomarkers needed to effectively manage the different BrCa subtypes.
- Circulating BrCa cells (CBrCaCs) are established prognostic biomarkers of early and metastatic BrCa. Additionally, their enumeration and characterization are useful in monitoring for treatment efficacy and disease relapse.

(continued)

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The ability to track CBrCaC clones with different genotypes from the primary tumor informs real-time treatment decision-making.

4.1 Introduction

Breast cancer (BrCa) is one disease that recent molecular and genetic research efforts have contributed immensely to the understanding of its pathology, and hence effective management. This feat has translated into improved survival outcomes. For example, the 5-year survival rate has improved from ~63 % in the 1960s to about 90 % in 2010. However, BrCa remains a disease of importance, being the second most common cancer in the world and the first and second most common cause of cancer-related deaths in the less and more developed parts of the world, respectively. Globally, 1.67 million cases were diagnosed and 522,000 people died from the disease in 2012. The 5-year global prevalence stands at 6.23 million. In the US, the expected incidence and mortality in 2016 are 249,260 and 40,890 cases respectively.

Early cancer detection offers the best opportunity for cure. For BrCa, the 5-year survival rate can be as high as 98 % for localized cancers, compared to a woeful 26 % rate for those diagnosed with advanced stage diseases. Late stage BrCa patients are treated with hormonal, chemotherapeutic, and biologically targeted agents, yet metastatic relapse occurs in a vast majority of cases, and this accounts for the poor prognosis. Also established in BrCa is the concept of field cancerization, which should enable the discovery of early detection biomarkers for BrCa.

Screening strategies, including mammography, have proven useful in early detection and improved survival of BrCa patients. Current screening efforts detect ~63 % of BrCas at an early stage. This is probably because mammography has limited utility in premenopausal women with dense breast tissue, and yet they contribute to about 12 % of all BrCas. Measurement of serum CA15-3 has also been helpful in the management of some women with metastatic BrCa. The need for novel accurate and validated biomarkers for early BrCa detection and management is hence acute, and there have been a plethora of biomarkers. But biomarkers in body fluids (circulation, breast fluid, urine, or saliva) should enable acceptable screening of all women at risk, especially premenopausal women and women in resource-poor communities of the world. Body fluid biomarkers will also fit into the ease of serial longitudinal sampling necessary for making important clinical decisions required for personalized BrCa oncology.

4.2 Screening Recommendations for BrCa

Screening for BrCa includes breast self-examination (BSE), clinical breast examination (CBE), and imaging primarily by mammography. A randomized case-control study concludes that BSE has limited value in early BrCa detection. However, because this is harmless, there is absolutely no reason why a woman should not occasionally examine her own breast or has her partner feel it for possible lumps. Breast self-examination should be performed monthly beginning at age 20. Clinical breast examination complements BSE and is useful in BrCa detection especially in younger women ($\leq$50 years). However, in comparison to mammography, CBE is less sensitive at cancer detection. This may be due to the limited experience of some family physicians and gynecologists in breast examination techniques. Also the improved detection by mammography is mostly in older women with less dense breast tissue. Clinical breast examination is recommended to be performed annually for women 40 years and older in conjunction with mammography. Mammography is currently the best screening modality for early detection of BrCa. In postmenopausal women with less dense breast tissue, mammography can reduce cancer mortality by at least a third. The method is, however, inaccurate when used in premenopausal women with dense breast mass. It is still useful for the fraction of premenopausal women with less dense breast tissue, for whom it is recommended. Radiation exposure and cost consideration must be examined in the use of this technology in all younger women. Body fluid biomarkers will be very useful for this group of women.

4.3 Molecular Pathology of BrCa

BrCa is a very heterogeneous disease, with different histopathologic and molecular subtypes. Knowledge on the molecular differences of BrCa informs adoption of efficacious targeted therapies in disease management.

4.3.1 Histopathologic Classification of BrCa

Histopathologic classification relies on the cytoarchitectural origins of the cancer, organization of the cancer cells, as well as demonstrating the presence or absence of substances secreted by the cancer cells. The well-defined and used method is that by the WHO. The various types are briefly described below:

- *Carcinoma in situ* comprises of neoplastic cellular proliferation that fills the lobular acini and ductules without breaching the underlining basement membrane. They are thus confined lobular and ductal lesions and can be completely

cured, by surgery, if these are the only malignant cells in the breast. Histoarchitectural compartmentalization of the transformed cells identifies two classes of in situ BrCa. Ductal and lobular lesions are referred to as ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS), respectively. Ductal carcinoma in situ is an impalpable lesion, but can be identified by mammographic screening as a cluster of microcalcification. Mammography often does not pick up LCIS. These are occult lesions diagnosed incidentally on biopsies performed for other concerns. Both lesions are risk factors for invasive BrCa, but DCIS can be a precursor of invasive disease.

- *Invasive or infiltrating lobular carcinoma* constitutes ~10 % of all invasive BrCas. This is a very challenging carcinoma both from a complete diagnostic workout, as well as treatment perspectives because of the mode of stromal invasion. The cells tend to file into the stroma in linear fashion (Indian file pattern). The often diffuse nature and lack of distinct boundaries make it difficult to accurately estimate tumor size or offer lumpectomy as a surgical alternative. The extent of invasion is thus more accurately determined using immunohistochemical stains.
- *Invasive ductal carcinoma* (IDC) accounts for the majority of BrCas, comprising between 60 and 80 % of all invasive BrCas. They are often associated with DCIS, partly explaining the belief that DCIS is a precursor lesion. They are further classified based on the degree of differentiation: being well differentiated (grade 1 tumors), of intermediate differentiation (grade 2 tumors), or poorly differentiated (grade 3 tumors). Approximately 10 % of IDCs are of special histomorphology and are thus diagnostically distinguished by special names. The vast majority are thus designated as "classic or not otherwise specified (classic/NOS)."
- The special IDC types (with defined histomorphology) include *mucinous or colloid carcinoma*, which comprises up to 2 % of all invasive BrCa. They are so named because the cancer cells produce extracellular mucin. These tumors are often diagnosed in older women and have good prognosis. *Medullary carcinoma* comprises ~5 % of all invasive BrCas. They resemble benign fibroadenomas with well-demarcated borders. They are associated with younger age and have a better prognosis than classic/NOS IDC. *Papillary carcinoma* comprises up to 2 % of invasive BrCa. They grow in a papillary fashion with cystic and solid compositions. They may be intraductal or invasive and have better prognosis than classic/NOS IDC. *Tubular carcinoma* make up another ~2 % of invasive BrCa. The tumors are well differentiated with tubular or small acinar growth patterns that resemble normal breast architecture. They may be associated with intraductal carcinoma and are in general small lesions measuring just about 1 cm. *Inflammatory carcinoma* is a very aggressive subtype of IDC. It is an inflammatory lesion with skin indurations and subcutaneous lymphatic obstruction that pulls on the underlying connective tissue ligaments resulting in an orange peel texture referred to as "peau d'orange." The remaining IDCs are very rare, comprising less than 1 % of all invasive BrCas. They include *adenoid cystic carcinoma* that contains cystic and glandular components and resemble

adenoid carcinoma of the salivary glands and *secretory (juvenile) carcinoma* that occurs in children and young adults. These tumors secrete lots of α-lactalbumin. *Apocrine carcinoma* as the name suggests resemble cutaneous sweat glands. *Metaplastic carcinoma* is composed predominantly of non-epithelial neoplastic cells. They could be bone, cartilage, or undifferentiated spindle cells (pseudosarcoma). *Cribriform carcinoma* cells are arranged in cribriform pattern and have a good prognosis. *Clear cell carcinoma* is diagnosed by distinct staining and has poor prognosis. *Paget's disease of the breast* is often associated with intraductal carcinoma with or without invasive components. Indeed, Paget's disease of the breast is the spread of cancer cells from the underlining lobe along the lactiferous dusts and sinuses and into the epidermis.

4.3.2 Molecular Classification of BrCa

In the era of personalized oncology, molecular classification is important not only for prognostication but selection of patents for targeted therapy. There are a number of targeted therapies for BrCa. Thus, testing for *ER*/*PR* and *HER2* status enables the deployment of the right treatment regimen. For example, tamoxifen and fulvestrant target ER-positive tumors, while trastuzumab and pertuzumab are useful in patients with HER2-positive tumors. There are other targeted agents that inhibit the PI3K (e.g., inhibition of mTOR with agents such as everolimus) and FGFR pathways.

Irrespective of the numerous and diverse histopathologic subtypes, molecular markers place all BrCas into just four subcategories. This is an important step because the vast majority (>80 %) of BrCa is histologically classified as IDC/NOS. Gene expression profiling and genomic analysis of chromosomal aberrations have enabled the molecular stratification of invasive BrCa into the four distinct subtypes (Table 4.1). They are HER2 enriched, luminal A, luminal B, and basal-like. Each subtype displays unique gene expression patterns as well as chromosomal abnormalities, which translate into treatment selection and prognostication.

The gene set that defines a group are those reminiscent of HER2-positive status, ER-positive status, breast cytoarchitecture (e.g., luminal and basal cells), or of the normal breast. For example HER2-enriched tumors express *ERBB2,* and *GRB7*, while luminal subtypes express *ESR1, GATA3*, and *PGR*, which are consistent with their HER2-positive and ER-positive status, respectively. Luminal tumors have a better prognosis than HER2-positive and basal-like tumors. But luminal B cases have worse outcome than luminal A. Similarly, basal-like tumors overlap considerably with triple-negative tumors and tumors harboring *BRCA1* mutations, and they express basal cytokeratin (CK5/6/17). The triple-negative tumors are not all basal-like, and a refined classification is needed for this subtype. They are common in younger and African-American women and are associated with worse prognosis. On the therapeutic front, the small molecule tyrosine kinase inhibitor (lapatinib)

Table 4.1 Features of the molecular subtypes of breast cancer

Tumors	Molecular features	Targeted therapy	Prognosis
Luminal A	*ER positive, HER2 negative* High-level amplifications at 8p11–12, 11q13–14, 12q13–14, 17q11–12, 17q21–24, 20q13 Gain at 1q, 16p Loss at 16q Express ESR1, PGR, GATA3 – consistent with ER status	Hormonal	Good
Luminal B	*ER positive, may be HER2 overexpressing* High-level amplifications at 8p11–12, 8q, 11-q13–14 Gains at 1q, 8q, 17q, 20q Losses at 1p, 8p, 13q, 16q, 17p, 22q	Hormonal	Worse than luminal A, but better than HER2 and basal-like tumors
HER2	*HER2 overexpressing, ER negative* High-level amplification of 17q Amplifications of 1q, 7p, 8q, 16p, 20q Loss of 1p, 8p, 13q, 18q Expression of ERBB2, GRB7 (17q loci)	HER2-targeted agents such as trastuzumab, lapatinib	Worse than luminal subtypes
Basal-like	*Triple negative – ER, PR, and HER2 negative* Locus amplification is uncommon Gains at 3q, 8p and 10p Losses at 3p, 4p, 4q, 5q, 12q, 13q, 14q and 15q Express CK5, CK17 (basal epithelial and myoepithelial genes) Loss of BRCA1 by methylation is common (hence share features with BRCA1 +ve tumors)	No established targeted therapy, but sensitive to epirubicin–cyclophosphamide combination therapy	Worse than luminal subtypes

and monoclonal antibody (trastuzumab) are efficacious in HER2-positive BrCas, while targeted hormonal therapies are offered for luminal tumors. Although there is currently no targeted therapy for basal-like tumors, they tend to be sensitive to epirubicin–cyclophosphamide combination therapy.

4.3.3 Clinically Relevant BrCa Transcriptomic Biomarkers

Global transcriptome profiling of BrCa is probably the best-illustrated example of the utility of "omics" data in cancer management. This area of research has received extensive discovery and valid biomarkers that are contributing to improved BrCa care. For instance, the molecular subtyping of invasive BrCa has important implications for objective management of this disease. In addition, validated gene sets that comprise the Oncotype DX® and MammaPrint BrCa tests have received FDA acceptance and are clinically available for companion diagnostic use. They both provide powerful prognostic and therapeutic predictions.

Oncotype DX® BrCa assay is a companion diagnostic test offered by Genomic Health. A carefully and rationally selected 21-gene set comprised of 16 BrCa genes and 5 control genes form this multigene predictive test. It interrogates the existing formalin-fixed and paraffin-embedded (FFPE) samples from patients using RT-PCR analysis, and in combination with a proprietary algorithm generates a recurrence score (RS) indicative of disease behavior and benefit from chemotherapy. The test, which is highly validated, is exempted by the FDA and endorsed by the American Society of Clinical Oncology® and the National Comprehensive Cancer Network® in making treatment decisions. The test is used for early stage (I and II), node-negative, hormone receptor-positive BrCa patients. Validated data indicate that patients at elevated risk of recurrence (those with high RS scores) benefit from adjuvant chemotherapy, while those with favorable prognosis (low RS scores) can just remain on hormone therapy and be spared the toxicities of adjuvant treatment.

Another personalized BrCa test in the clinic is MammaPrint, developed by Agendia. The 70-gene set that comprises this test were uncovered by scientists at the Netherlands Cancer Institute and has since then been well validated for stratifying early stage BrCa women into high and low risk for distant recurrence following surgery. MammaPrint is the first of these tests to be approved by the FDA in accordance with its IVDMIA guidelines. It is indicated for women with node-negative stage 1 or stage 2 invasive BrCa irrespective of ER status. Similar to Oncotype DX® BrCa test, MammaPrint identifies patients who will benefit from adjuvant therapy, because patients with predicted unfavorable benefit from such therapy are the low-risk category. Unlike Oncotype DX®, the MammaPrint® BrCa test requires high-quality RNA, either fresh frozen samples or those collected in RNA preservatives.

Other BrCa gene expression signatures with prognostic and predictive values include the HOXB13:IL17BR ratio (H/I index) and the 76-gene signature assays. The H/I index assay was discovered through global gene expression profiling of ER-positive early stage BrCa from women treated with adjuvant tamoxifen. The two genes were identified to be the best predictor of disease recurrence. It has received extensive independent validation studies involving multiple samples. High H/I ratio is associated with tumor aggressiveness, worse outcome, and tamoxifen failure. The performance of the assay is independent of tamoxifen treatment and is

much better in early stage lymph node-negative tumors. The test, which is performed on FFPE samples, is offered by bioMérieux (bioTheranostics). The 76-gene signature was discovered by scientists at Rotterdam and has been validated as a potential strong prognostic indicator in premenopausal and postmenopausal women with ER-positive BrCa. This assay requires high-quality RNA for analysis (frozen or preserved in RNA protective reagents).

4.3.4 Molecular Model of BrCa Progression

Molecular data provide a not so distinct complex pathway of BrCa progression. This progressive model involves epigenetic alterations, mutations, and importantly chromosomal instabilities that are modified by individual genetic composition. The high level of BrCa heterogeneity precludes charting a simple and well-defined model as is in colorectal cancer. An even more complex issue is the evidence that the sorts and levels of genomic damage that drive BrCa also depend on the normal progenitor cell type of origin.

Hormonal stimulation, gene promoter hypermethylation, loss of proliferation control, increased cell cycle activity, lack of checkpoint control, loss of DNA repair mechanisms, loss of cell death control, telomerase expression, eroding telomeres (telomere crisis), high genomic instability, loss of tumor suppressor gene function through mutations and methylation, oncogene amplification, and growth factor secretion are among the factors that operate in concert to drive the normal breast epithelial cell to hyperplasia, through in situ carcinoma then to invasive carcinoma. Analysis of deletions, amplifications, and recurrent mutations reveal relevant pathways in BrCa progression, including the ERBB2/EGFR and PI3K signaling pathways.

In spite of the difficulties encountered in charting a path for BrCa development, it is recognized that ER status has distinct molecular pathology that is relevant to disease progression. Estrogen receptor-expressing tumors harbor deletions at 16q and gains of 1q, while ER-negative tumors have increased genomic instability and loss of *BRAC1* and *TP53,* with amplification of *HER2*. With the demonstration that ER-positive tumors may progress from low-grade in situ to high-grade tumors, attempted progressive models are provided for ER-positive and ER-negative tumors (Fig. 4.1).

4.4 Circulating BrCa Biomarkers

There has been extensive pursuit in uncovering circulating BrCa biomarkers. This effort has led to the discovery of a plethora of molecular genetic alterations in women with BrCa. Validations of most of these biomarkers are awaited. However, circulating breast cancer cell characterization is in routine clinical practice.

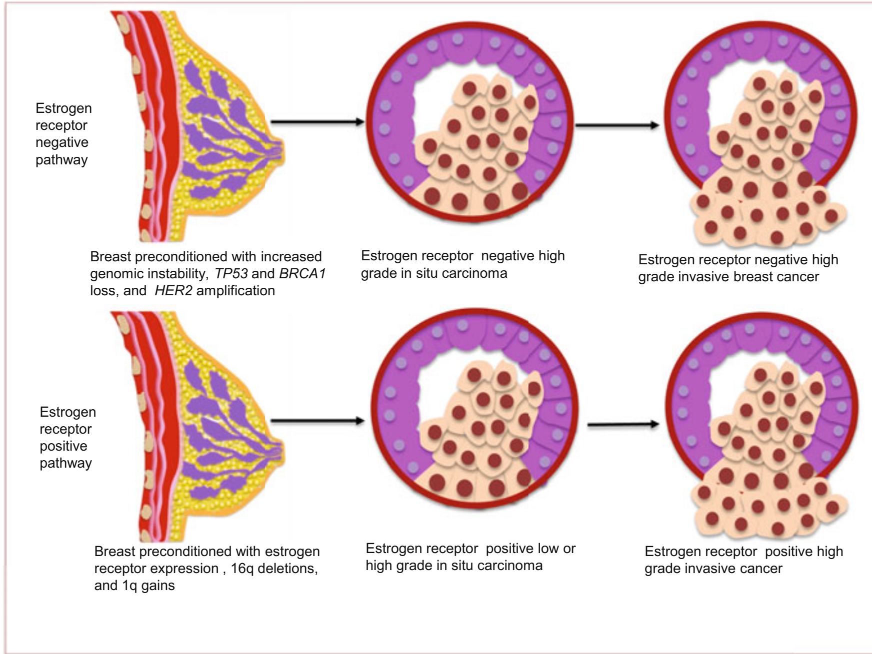

Fig. 4.1 BrCa molecular progression model based on estrogen receptor (ER) status

4.4.1 Circulating Cell-Free Nucleic Acid Content as BrCa Biomarkers

Attempts have been made to uncover cell-free DNA as clinical biomarkers of BrCa. Serum analysis of DNA fragments of ALU repeats in women with primary BrCa and healthy female controls indicate a mean higher DNA integrity index (DII) in stages II, III, and IV cancer patients than controls with discriminatory AUROCC of 0.79. DII correlated with size of invasive cancer and was significantly higher in those with lymphovascular and lymph node metastasis. Detection of lymph node metastasis was accurate, with AUROCC of 0.81. In a multivariate analysis, lymphovascular invasion and DII significantly predicted lymph node metastasis, making this a promising biomarker for the detection of BrCa progression and lymph node involvement [1]. Sunami et al. analyzed the sera of BrCa patients for LINE1 DNA fragments to predict possible early detection of BrCa [2]. These fragments were increased in BrCa patients, and the copy number correlated with tumor size. Deligeze et al. proved that non-apoptotic DNA fragments contribute to the change in DNA levels during adjuvant chemotherapy of BrCa [3]. Larger DNA fragments released from non-apoptotic cells mainly contributed to the elevated DNA levels in circulation during adjuvant chemotherapy [4]. Another study compared ccfDNA with CTCs, CA15–3, and current standard of care (medical imaging) and found

unequivocal evidence for the use of ccfDNA in BrCa monitoring [5]. Circulating cell-free DNA was demonstrated in 97 % of women. Concordance between tumor mutations and mutations in plasma were observed. Importantly, some mutations detected in plasma DNA were not found in archival tissue samples collected 10 years prior, indicating the presence of tumor evolution with time. For diagnostic performance, levels of ccfDNA performed at 96 % compared to the CELLSEARCH® system for CTCs that achieved 87 % and levels of CA15–3 that performed at 78 % in BrCa detection among these women. Increasing plasma levels of ccfDNA was a better prognostic indicator than conventional imaging assessment. The increasing ccfDNA levels were associated with poor overall survival (OS), and detected progressive breast disease 5 months before conventional imaging could do so, making this a much early detection assay.

4.4.2 Circulating BrCa Epigenetic Biomarkers

Alterations in the epigenome, especially promoter CpG island hypermethylation, are prevalent in BrCa samples. Although these tumor markers are yet to be clinically validated and translated, the promise to use these biomarkers in early detection and prognostication is not far from reality. Of even more clinically relevant is the sensitive detection of these cancer-specific epigenetic changes in minimally invasive samples including blood, nipple aspirate fluids, ductal lavage, and fine needle aspirates from patients. Several methylated genes detected in body fluids are potential biomarkers of early BrCa detection. Whereas these genes demonstrate variable sensitivities, they appear to be very specific (up to 100 %) for cancer. Promoter hypermethylation of several genes including *RASSF1A*, *APC*, *CDKN2A*, *CDH1*, *CCND2*, *HIC-1*, *DAPK*, *GSTP1*, *SFN* (*stratifin*), *RAR-β*, and *TWIST* occur at various frequencies in BrCa samples.

4.4.2.1 Circulating BrCa Diagnostic Epigenetic Biomarkers

As a proof of concept, Silva et al. were able to demonstrate that de novo methylated cancer genome could be detected in circulation of BrCa patients [6]. Exon 1 of *CDKN2A*/*INK4A* hypermethylation was detected in 23 % of tumors and specifically in 14 % of paired plasma samples. Rykova et al. demonstrated using methylation of *RASSF1A* and *HIC-1* that cancer-derived circulating DNA is primarily cell surface bound (on RBCs and WBCs) [7]. In this study, detection of gene methylation was enhanced when DNA was eluted from cell surfaces (csb-DNA), and was even positive in samples that were negative for methylation in plasma. A follow-up study of methylation of *RASSSF1A*, *RARβ2* and *CCND2* indicated positive detection in 13 % of breast fibroadenomas, and in 60 % of women with BrCa. Using csb-DNA as template increased the detection of methylated genes to 87 % for

adenomas and 95 % for BrCas [8]. But this assay shows no specificity for cancer, as it detects adenomas as well.

Serum was examined as a less invasive sample (compared with NAF/DL) for BrCa methylation biomarker discovery and utility. Three important genes involved in cancer biology (*RASSF1A*, *APC,* and *DAPK*) were selected for this study. Promoter hypermethylation in one or more genes was observed in 94 % of tumor DNA (*RASSF1A* at 65 %, *DAPK* at 50 %, and *APC* at 47 %) and 76 % of corresponding serum samples. These findings also included premalignant and early stage disease (DCIS, LCIS, and stage I) patients, attesting to the early detection potential. Specificity of this assay was 100 %, because all samples from control individuals (normal, inflammatory, and nonneoplastic breast disease patients) were negative [9]. Shukla and colleagues further investigated *RASSF1A*, *RARβ2*, and *HIC-1* methylation in total ccfDNA, which indicated the absence of methylation in healthy controls but in as many as 95 % of BrCa patients as well as patients with benign breast disease (fibroadenomas at 60 %) [10]. The methylation status of *RASSF1A* and *RARβ2* in invasive BrCa tissues and paired sera has revealed the presence of *RASSF1A* methylation in 85 % of breast tumors and 75 % of paired sera [11]. *RARβ2* was methylated in just 10 % of tumor samples [10].

A study attempted to address an important need of accessible diagnostics for underserved population of women. It focused on BrCa from West African women, a geographic region where mammography and other sophisticated diagnostics are not readily available. Methylation of *APC*, *GSTP1*, *RASSF1A*, and *RARβ2* in plasma from 93 women with primary BrCa and 76 controls were assessed. Cutoff values and gene panel selection were achieved using ROC curves in a training data set, and assay performance was tested on a validation set. A sensitivity of 62 % and specificity of 87 % was achieved. Importantly, 33 % of early stage cancers were detected by this serum assay [12].

In order to uncover an early detection biomarker for women at an above-average risk, a study from the BrCa family registry focused on *RASSF1A* methylation in plasma of BrCa women. Blood samples were collected prior to diagnosis, and cases were tightly matched with controls. Eighteen percent of plasma samples were positive for *RASSF1A* methylation, and two healthy high-risk women were also positive for methylation. None of the population-based healthy control plasma samples were positive. All available tumor samples were positive for *RASSF1A* methylation. This finding could serve as an early warning sign for this population at above-average risk for BrCa [13]. Another study targeting hypermethylation of *RASSF1A* and *DAPK1* in sera from BrCa patients, healthy women, and those with benign breast disease found hypermethylation of at least one gene in 96 % of cancer, 43 % of benign disease patients, and 8 % of controls. *RASSF1A* and *DAPK1* were methylated at frequencies of 69 % and 88 %, respectively, and both gene methylations were associated with ductal carcinomas [14].

The methylation status of other genes in circulation as diagnostic biomarkers of BrCa has been evaluated. The diagnostic value of *APC* hypermethylation was assessed in 84 women with BrCa. *APC* hypermethylation was detected in 45.2 % of tumors and 31 % of paired plasma samples. A statistically significant correlation

existed between methylation in tissues and paired serum samples. The use of this serum assay as a single biomarker for BrCa detection performed at a sensitivity and specificity of 68.4 % and 97.8 %, respectively [15].

Sharma et al. attempted to find noninvasive diagnostic biomarkers for a population of Indian women with invasive BrCa. They initially examined *CDKN2A/INK4A*, *CDKN2A/ARF*, *CCND2*, and *SLIT2* methylation in invasive BrCa and paired sera as a possible screening tool [16]. Methylation of at least one gene was observed in 86 % and 83 % of tumor and sera, respectively. Each gene was methylated at different frequencies, and there was concordance between methylations in tissue and sera. In another study by this group [17], promoter methylation of the following tumor suppressor genes, *TMS1*, *BRAC1*, *ERα*, and *PRB*, was evaluated in samples from a population of Indian women where invasive BrCa is engulfing younger women and often with poor prognosis (ER-/PR-negative tumors). Methylation in at least one gene was present in 72 % of tumors and 64 % of paired sera. Methylation of three genes could be detected in as many as 34 % of tumors and 24 % of associated sera. Methylation in tumor and sera for each gene was concordant, and methylation of *ERα* and *PRB* were significantly correlated. A follow-up study examined methylation of *SFN*, *ERα*, and *PRB* in tumor and circulating blood of 100 BrCa women. Methylation of *SFN* in tumors (61 %) correlated significantly with sera (56 %). Significant correlations were also found for methylation of *ERα* with *PRB* and *SFN* [18].

4.4.2.2 Circulating BrCa Prognostic Epigenetic Biomarkers

The methylation of *RASSF1A* in circulation is a potential predictive biomarker of response to adjuvant tamoxifen systemic therapy. Methylation frequencies in pre- and posttreatment sera were identical (19.6 % pretreatment and 22.3 % posttreatment). The presence of *RASSF1A* methylation after 1 year of surgery and adjuvant chemotherapy was a predictor of poor outcome with a relative risk (RR) of 5.1 for relapse and 6.9 for death. The loss of serum *RASSF1A* methylation during treatment was indicative of response [19]. Muller et al. examined 39 genes in an evaluation set using high-throughput MethyLight assay [20]. In applying appropriate selection strategies, five genes (*ESR1*, *APC*, *HSD17B4*, *HIC*, and *RASSF1A*) were identified to be of prognostic potential. Methylation of *RASSF1A* and/or *APC* was found to be associated with poor outcome in the training set and was confirmed in a validation cohort. Multivariate analysis of all pretreatment sera confirmed *RASSF1A* and *APC* methylation to be an independent predictor of poor outcome with an RR 5.7 for death. In a subsequent study, Muller et al. examined the prognostic value of methylation in several genes in pretreatment sera from patients with breast ($n = 112$) and cervical ($n = 93$) cancers [21]. In cervical cancer, hypermethylation of *MYOD1*, *CDH1*, and *CDH13* were significantly associated with poor outcomes. This report further confirmed the importance of serum hypermethylation of *RASSF1A* and *APC* as independent prognostic biomarkers for BrCa.

DNA from tumor, normal breast tissue, normal blood cells, and plasma were used for genetic analysis targeting MSA using the following markers, D17S855, D17S654, D16S421, TH2, D10S197 and D9S161, as well as *TP53* mutations and methylation of *CDKN2A*. At least one of these alterations occurred in tumors from 90 % of patients and in 66 % of plasma samples. The presence of cancer genome in circulation evidenced by these molecular alterations correlated significantly with poor prognosis [22]. In another study, 44 % of patients harbored tumor DNA alterations (either MSA, *TP53* mutation, or *CDKN2A* methylation). These alterations were detected in plasma before mastectomy, and 19.5 % retained plasma gene alterations 4–6 weeks after mastectomy, and this was significantly associated with patients who presented with >3 lymph node involvement, vascular invasion, and higher histologic grade at diagnosis. These findings have potential for prognostication at initial diagnosis [23]. Hypermethylation of *CDKN2A* and *CDH1* were examined in tumor and plasma samples and correlated with two serum markers, CEA and CA 15–3 in women with invasive BrCa. *CDKN2A* was methylated in 11 % of tumors and 8 % of plasma samples, while *CDH1* methylation was in 25 % of tumors and 20 % of plasma samples. Either gene was methylated in 31 % of tumors, but in as many as 82 % of plasma samples from those with gene methylation. Methylation of *CDKN2A* in tumor was associated with advanced stage, size, and nodal metastasis; however, plasma *CDKN2A* methylation only correlated with nodal metastasis. Combined *CDKN2A* methylation and serum CEA levels were significantly associated with tumor stage, size, and extensive nodal involvement [24]. The promoter methylation of DNA repair genes (*BRAC1*, *MGMT,* and *GSTP1*) in tumor and circulating DNA of 100 BrCa patients were examined. These genes were methylated at various frequencies from 27 to 32 % in both tissue and sera and were associated with loss of protein expression (except for *BRAC1*). *GSTP1* and *BRAC1* hypermethylation were independent predictive factors for disease recurrence [25].

4.4.3 Circulating BrCa Genetic Biomarkers

4.4.3.1 Circulating Chromosomal Alterations as BrCa Biomarkers

BrCa is a disease of chromosomal instability including LOH, aneuploidy, as well as epigenetic alterations and mutations of specific genes. Together, these lesions interfere with normal functioning of signaling pathways important in cellular maintenance such as apoptosis, DNA repair, senescence, cell cycle control, cellular proliferation, and differentiation among others. Several chromosomal regions are amplified, deleted, or rearranged in BrCa. Affected chromosomes include 1, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, 20, and 22. That these instabilities are causative and/or drivers of BrCa progression is the fact that benign breast pathologies such as fibroadenoma rarely harbor these genetic alterations, with premalignant cells containing intermediate proportions of chromosomal instabilities, but

levels become markedly increased in aneuploid BrCas. Mutations and other DNA sequence anomalies are found in *BRAC*, *TP53*, *EGFR*, *VEGF*, and *HER2* and multiple other genes in BrCa. These genetic alterations have been profiled in body fluids as BrCa biomarkers.

4.4.3.2 Circulating Mutated Genes as BrCa Biomarkers

Mutations in the guardian of the genome, *TP53* have been detected in ~37 % of primary BrCa tissues. Of these positive samples, 46 % had detectable mutations in ccfDNA in plasma. The mutations correlated with clinical stage, tumor size, estrogen receptor status, and lymph node metastasis. A 29-month follow-up revealed in both univariate and multivariate analyses that *TP53* mutations (in both plasma and tissue) significantly predicted recurrence-free and overall survival. In both lymph node-positive and lymph node-negative patients, mutation status still conferred a worse survival outcome [26]. Mutations in *TP53* and *CDKN2A* as well as six microsatellite markers were assayed in tissues, plasma, and blood cells in women with BrCa before and after mastectomy. Forty-four percent of the genetic alterations in tissue samples were detectable in plasma prior to mastectomy. Plasma served as a useful noninvasive medium to monitor women who after surgery still harbored micrometastatic cancer cells [23]. An ultrasensitive method referred to as MIDI-Activated Pyrophosphorolysis (MAP) was designed to detect microinsertions, deletions, and indels (MIDI) that make up 15 % of all genomic mutations. Using this method, plasma samples could be assayed to determine responders and nonresponders to therapy. Posttreatment remission was associated with undetectable *TP53* mutations as opposed to non-responders who had persistent circulating mutant *TP53* [27]. *PIK3CA* mutations were examined in ccfDNA using BEAMing technology. The detection rate was about 29 % in two independent cohorts, and this assay achieved a 100 % concordance between plasma and tissue samples. Cancer recurrence altered *PIK3CA* status, thus requiring the reassessment of patients using body fluid samples such as blood [28].

4.4.3.3 Circulating MSA as BrCa Biomarkers

Microsatellite alterations (MSAs) in BrCa tissue samples correlate with circulating levels and are detectable in both early and advanced stage disease. Seven polymorphic markers were used for LOH analysis in primary and metastatic BrCa samples, as well as matched blood and bone marrow plasma. Marker D3S1255 showed concordant frequencies of LOH in tissue, blood, and bone marrow plasma and also was the marker with the highest frequency of LOH in serum and bone marrow plasma. Tumor tissue samples were more frequently altered at D13S2118 and D17S855. A significant relationship between lymph node-positive status and LOH in serum samples was observed for marker D3S1255, and this marker in

tissues correlated with undifferentiated nuclear grade [29]. MSI and LOH (used multiple markers including markers DM1 and D17S1325) in plasma or sera from BrCa patients indicated LOH was present in 15–48 % of plasma samples [30]. The LOH was importantly detectable in patients with early stage disease, such as those with small tumors (T1), low grade I, and in situ carcinomas, indicating their potential as noninvasive early detection biomarkers. Two polymorphic markers, DM1 and D16S400, were used to assess LOH and MSI in plasma, tissue, and lymphocyte DNA from primary and metastatic BrCa patients [31]. Plasma LOH (31.3 %) and MSI (11.6 %) were frequent events in BrCa patients, and these changes were concordant with tissue MSI. MSA was detectable in women with primary and metastatic BrCa. Plasma DNA was found preferable to bone marrow or other invasive sampling methods to assess tumor burden in sequential sampling for BrCa management.

4.4.4 Circulating BrCa Coding RNA Biomarkers

Transcripts of telomerase, *hTR* and *hTERT,* the two components of telomerase, were assayed in sera from patients with BrCa, benign breast disease, and in healthy volunteers. Transcripts of *hTR* were present in 94 % of tissue samples but in only 28 % of sera, while *hTERT* was positive in tissue samples at the same frequency of 94 % and in sera of 25 % of patients. However, they were undetectable in sera from patients with benign breast disease and healthy controls [32]. Novakovic et al. also addressed the possible presence of *hTR* and *hTERT* transcripts in plasma from women with primary BrCa and patients with advanced melanoma and advanced thyroid cancer [33]. Among the BrCa cohort, *hTR* transcripts were present in all tested plasma, but *hTERT* was present in 52.2 % of the samples. In malignant melanoma patient samples, *hTR* and *hTERT* were detected at frequencies of 100 % and 71 %, respectively.

Transcripts from other genes have been assayed in circulation of BrCa patients as potential prognostic biomarkers. Yie et al. tested *BIRC5* expression in BrCa cells in circulation [34]. Of 67 patients tested, 34 (50.7 %) expressed the gene compared to none of the 135 controls. *BIRC5* expression was associated with blood vessel invasion; histologic grade; tumor size; nodal status; ER, PR, and HER2 status; and clinical stage. In a 26-month follow-up period, 81.8 % of patients with positive *BIRC5* expression relapsed compared to 33.3 % of patients without initial *BIRC5* expression. Polycomb member, BMI-1, controls cellular proliferation, and its deregulation is associated with cancer. Because deregulated expression is demonstrated in many tumor tissues, analysis in plasma was conducted. The expression of *BMI-1*, examined in plasma from 111 BrCa patients and 20 controls, revealed elevated expression in samples from cancer patients. This high expression correlated with poor prognostic factors such as *TP53* expression and PR-negative status. In patients with advanced stage disease, plasma *BMI-1* transcript levels also correlated significantly with poor disease-free and OS [35]. This group later

examined the predictive value of plasma *CCND1* and *TYMS* (*thymidylate synthase*) mRNA in BrCa women. Among patients clinically classified as having good prognosis, *CCND1* expression in plasma conferred poor outcome. The presence of both markers in plasma was associated with dismal response to therapy upon relapse. Additionally, *CCND1* expression predicted patients nonresponsive to tamoxifen [36]. Serum *metastasis* mRNA as a predictive biomarker was investigated. As a screening tool, this assay achieved a sensitivity of 85 % and specificity of 100 % for BrCa detection. Prognostically, higher serum levels were associated with lymph node involvement and poor survival (six times worse outcome compared to those with low levels). Serum *metastatin*-positive patients had distant metastasis, while patients who were negative had only local recurrences [37].

4.4.5 Circulating BrCa Noncoding RNA Biomarkers

Breast cancer is characterized by deregulated expression of hundreds of miRNAs that participate in disease progression. Among these are upregulated miR-10b, miR-21, and miR-27a, as well as downregulated let-7, miR-125a, miR-125b, and miR-206. Indeed, miRNA-deregulated expression characterizes various neoplastic cells of the breast. For example, miR-15b, miR-21, miR-30d, miR-141, miR-183, miR-200b, and miR-200c are upregulated, while miR-572, miR-638, miR-671-5p, and miR-1275 are downregulated in atypical ductal hyperplasia. Some of these miRNAs including miR-21, miR-141, miR-183, miR-200b, miR-200c, miR-638, and miR-671-5p show similar deregulated expression patterns in DCIS/IDC. Moreover, some miRNAs characterize molecular BrCa subtypes. For instance, miR-214 is elevated in normal-like, luminal A, and triple-negative BrCa subtypes. Noteworthy, aberrant expression of miRNAs is involved in BrCa stem cell proliferation, self-renewal, differentiation, and metastasis. Among these BrCa stem cell miRNAs are upregulated miR-27 (induced by VEGF), miR-142, miR-214, and miR-221/222 clusters and decreased expression of let-7 family, miR-183 and miR-200 clusters. Several of these as well as novel miRNAs show differential levels in the peripheral circulation of BrCa patients, with diagnostic and prognostic relevance (Table 4.2).

4.4.5.1 Circulating BrCa Diagnostic miRNA Biomarkers

Zhu and colleagues were first to publish altered serum miRNA levels in BrCa patients [38]. Levels of miR-16, miR-145, and miR-155 conferred risk for BrCa, and miR-155 correlated with PR status. Circulating miR-148b, miR-376c, miR409-3p, and miR-801 are uncovered as early detection BrCa biomarkers. Indeed, except for miR-801, the rest could differentiate BrCa from benign tissues [39]. Low plasma levels of miR-145 and increased miR-451 discriminate BrCa from controls [40]. Serum miR-145 is rather elevated in some cancer patients [41]. Other potential early detection circulating BrCa miRNAs are miR-21, miR-92a, miR-10b,

Table 4.2 Circulating BrCa miRNAs

Diagnostic		Prognostic
Increased	Decreased	Increased
miR-10b, miR-16, miR-21, miR-25, miR-29a, miR-34a, miR-125b, miR-141, miR-145, miR-148b, miR-155, miR-191, miR-200a, 200b, miR-200c, miR-203, miR-210, miR-215, miR-222, miR-299-5p, miR-324-3p, miR-373, miR-375, miR-376c, miR-382, 409-3p, miR-411, miR-451, miR-801	miR-30a, miR-92a, miR-145, miR-768-3p	let-7a, miR-10b, miR-34a, miR-141, miR-155, miR-195, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375, miR-801

miR-125b, miR-155, miR-191, miR-382, and miR-30a [41–43]. Circulating miR-195 was diagnostic at 87.7 % sensitivity and 91 % specificity. MiR-195 was also detected in blood from early stage cancer (tumors < 2 cm) patients, and levels correlated with tumor size. The levels of miR-195 returned to normal after surgery [44]. A panel of miR-16, miR-25, miR-222, and miR-324-3p was highly accurate (sensitivity of 92.1 % and specificity of 93.4 %) in detecting BrCa. Serum miR-29a and miR-21 are elevated in BrCa patients as well, and serum miR-155 is a potential diagnostic biomarker of BrCa.

Some miRNAs may demonstrate ethnic variation, and this needs to be considered in miRNA studies. There are noted ethnic variations in BrCa genetics, indicated by some biomarkers such as mtDNA alterations. A study of circulating miRNA identified such variation as well. Zhao et al. found that African-American and Caucasians had different miRNA profiles compared to their ethnic-matched controls [45]. But miR-181a and miR-1304 were common to both ethnic groups. Guo et al. found decreased levels of miR-181a in women with BrCa [46].

Evidence-based diagnostic utility of circulating BrCa miRNA has been revealed by meta-analytical studies. Two meta-analyses achieved similar performances. Lui et al. included 31 studies from 16 published works that involved 1668 cases and 1111 controls. A pooled sensitivity of 77 %, specificity of 88 %, PLR of 4.2, NLR of 0.29, DOR of 18, and SAUROCC of 0.89 were achieved [47]. Cui et al. included 15 studies involving 1368 BrCa patients and 849 controls. The sensitivity, specificity, and AUROCC from their analyses were 82 %, 82 %, and 0.9217, respectively [48]. Both studies found better diagnostic performances with the use of panel rather than single miRNAs as biomarkers. Specific miRNAs associated with BrCa detection have also been determined by meta-analyses. Lv et al. found miR-10b, miR-21, miR-155, and miR-222 were often deregulated in BrCa and hence are suggested as potential BrCa diagnostic biomarkers [49]. Focusing on circulating miR-155, three high-quality studies (QUADAS scores 12 or 13) involving 184 patients and 75 controls achieved a pooled sensitivity of 79 %, specificity of 85 %, and SAUROCC of 0.9217 for BrCa diagnosis [50]. Efforts at validating these miRNAs in circulation will augment BrCa detection, especially in women deprived of, or not suitable for mammographic screening.

4.4.5.2 Circulating BrCa Prognostic miRNA Biomarkers

A number of studies have explored the prognostic relevance of circulating miRNA in BrCa patients. Some circulating miRNAs can discriminate primary from metastatic BrCa, and miR-10b, miR-34a, and miR-155 can predict the presence of metastatic disease [51]. MiR-34a, in particular, is associated with advanced stage disease. MiR-215, miR-299-5p, and miR-411 are elevated in sera and tissue samples from metastatic BrCa patients compared to controls [52]. In other studies, CTC status, which is a prognostic marker for BrCa, correlated with circulating miRNAs. In addition to differentiating metastatic BrCa from controls, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375, and miR-801 levels correlated with CTC status. MiR-200b alone could predict progression-free and overall survival and even performed much better than CTC counts as a prognostic biomarker [53]. MiR-10b and miR-373 may control BrCa metastasis, and circulating levels are associated with lymph node involvement [54].

4.4.6 Circulating BrCa Protein Biomarkers

4.4.6.1 Circulating Protein Spectral Peaks as BrCa Biomarkers

Several proteomic approaches have used mass-to-charge ratios (*m*/*z*) primarily as discovery biomarkers for BrCa. SELDI MS has been used to uncover several circulating peptide peaks that can discriminate women with BrCa from healthy controls. A profiling of serum samples identified a single peak at *m*/*z* 2790 that modulated with paclitaxel or adjuvant 5-fluorouracil, adriamycin, and cyclophosphamide (FAC) chemotherapy [55]. Five peaks from this study discriminated between cancer and controls, and these peaks remained even after surgery, possibly due to the presence of micrometastasis [55]. Serum proteomics and application of artificial neural network was used to discover diagnostic protein BrCa biomarkers. In this study, an intact diagnostic model using 253 discriminatory peaks and a refined model using only the top four peaks had similar diagnostic accuracies. The intact model had a sensitivity of 83.3 % and specificity of 88.9 %. Performance for the top four peaks was 76.5 % and 90.0 %, respectively, for sensitivity and specificity [56]. Seven ion peaks were identified in a training set, and these achieved a sensitivity of 95.6 % and a specificity of 86.5 % in blind test set. When applied to a validation set samples obtained 14 months later, the sensitivity and specificity were 96.5 % and 85.7 %, respectively, and these peaks identified all early stage (T1a) tumors [57]. Sera from patients with familial and sporadic BrCa, as well as healthy controls, revealed shared peaks at *m*/*z* 11,730 and 5066 between both cancer groups. However, a unique peak at *m*/*z* 8127 only appeared in samples from familial BrCa patients [58].

A study that compared two methods of pre-analytical sample preparation, direct complete serum analysis and analysis after membrane filtration (50 kDa cutoff) to

remove high molecular weight proteins was conducted. Complete serum produced eight peaks with 81 % sensitivity and 72 % specificity for BrCa detection. Membrane-filtered samples had four spectral components with 80 % and 81 % sensitivity and specificity, respectively, which is a modest improvement over direct complete serum analysis [59]. Fourteen discriminatory peaks achieved a sensitivity of 89 % and a specificity of 67 % for BrCa early detection [60]. The use of ClinProt kit combined with MS and support vector machine algorithm was able to detect three peaks at *m/z* 698, 720, and 1866 that could detect BrCa with a sensitivity and specificity of 91.89 % and 91.67 %, respectively [61].

MALDI-TOF MS was used in a case–control study to identify BrCa diagnostic serum proteome biomarkers. Seventy-two peaks with significant discriminatory ability were noted. In a cross validation study, these peaks had a sensitivity and specificity of about 85 % for early detection of BrCa [62]. Callesen et al. then performed a systemic review to ascertain the reproducibility of MS proteome data. Of 20 inclusive publications from 1995 to 2006, only three had protein sequence identified with the remainder reporting only discriminatory peaks. In spite of known problematic issues with such data, 45 % of the peaks were concordant with Callesen's data, of which 25 % could potentially be used to detect BrCa [63].

After removal of high molecular weight proteins (albumin, Ig), sera from stage I/II BrCa patients and matched controls were subjected to MALDI-TOF MS. Three spectral components at *m/z* 2303, 2866, and 3579 Da were identified that had sensitivity and specificity of 88 % and 78 % for early BrCa detection [64]. Two serum processing procedures, weak cation exchange (WCX), and reverse-phase C18 (RPC18) magnetic beads were applied to samples prior to MALDI MS analyses. This approach generated two data sets. Double cross validation revealed a sensitivity and specificity of 82 % and 87 % for the WCX method and 73 % and 93 % for the RPC18 samples. When both were combined, the sensitivity and specificity improved to 84 % and 95 %, respectively [65]. The search for pretreatment serum peptide biomarkers predictive of axillary lymph node metastasis is needed for prognostic stratification. To identify such biomarkers, MALDI MS was used to screen node-negative and node-positive, as well as control group samples. Multiple discriminatory peaks occurred between cancer and controls and between node-positive and node-negative cohorts. Peaks at *m/z* 5643, 4651, 2377, and 2240 as a panel performed at a sensitivity of 87 % and a specificity of 87.2 % and an accuracy of 87.1 % in differentiating node positive from negative patients [66]. Eight spectral peaks differed between presurgical and postsurgical BrCa patients. Four peaks separated postsurgical relapsed patients from those without relapse. A peak at *m/z* 3964 was lost after surgery and reappeared following relapse, indicating its potential as a biomarker for detecting early relapse [67].

SELDI serum profiles correctly classified 87 % of *BRAC1*-positive women who developed BrCa from those who did not. Also 81.5 % of lymph node-positive and 77.5 % of node-negative patients were correctly classified [68]. This group further examined *BRAC1* carrier prediction data, and a sensitivity and specificity of 87 % each was achieved in differentiating *BRAC1* carriers who developed cancer from those who did not. In regard to sporadic cancer, the sensitivity was 94 % and

specificity 100 % in differentiating *BRAC1* patients who developed cancer from women who developed sporadic cancer. The profiles of *BRAC1* patients without cancer was similar to controls [69].

4.4.6.2 Circulating BrCa Proteomic Biomarkers

Circulating Anti-BrCa Antibodies as BrCa Biomarkers
Circulating immune proteins have been assayed in BrCa patients. Sera from BrCa patients and controls were reacted with proteins from SUM-44 BrCa cell line and individual sera analyzed for primary antibodies. Reactivity against three proteins was identified as isoform of novel oncogenic protein that regulates RNA-protein interaction (named RS/DJ-1). This was observed in patients and not control samples. RS/DJ-1 is secreted by SUM-44 cells and detectable in sera of 37 % of BrCa patients [70]. Serological proteome analysis (SERPA) was used to identify antigens of infiltrating ductal carcinoma (IDC). Sera from patients and controls were treated with MCF-7 cell line protein extracts, and immunoreactive proteins were isolated and subjected to MALDI MS. Twenty-six antigens reacted with sera from BrCa patients, and these included HSP60, prohibitin, β-tubulin, haptoglobin-related protein, peroxiredoxin-2, hnRNPK, Mn-SOD, and F1-ATPase [71]. A follow-up study by this group using SERPA immunoproteomics identified an immunoreactive protein in sera of over 50 % of patients with IDC. This protein was isolated as elongation factor-Tu, which needs further characterization [72]. Antibody microarray targeting acute phase proteins and complement factors uncovered six proteins that significantly differed between BrCa and healthy control samples. An artificial neural network analysis performed at 69 % sensitivity and 76 % specificity for cancer detection [73].

Circulating ITIH4 as BrCa Biomarker
Inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4) is a type II acute phase protein that is involved in inflammatory responses, liver development, and regeneration. It is secreted into the circulation and cleaved by plasma kallikrein into two smaller subunits. Proteomic studies have identified this molecule as a potential cancer biomarker. SELDI MS analysis of samples from all stages of BrCa identified three peaks, one at 4.3 kDa that was decreased and two at 8.1 kDa and 8.9 kDa that were elevated in patient samples. These peaks had a sensitivity of 93 % and a specificity of 91 % for BrCa detection [74]. The above discriminating peaks, designated BC1, BC2, and BC3, were identified as ITIH4, (BC1 4.3 kDa), complement component C3a (BC3 8.9 kDa), and C-terminal truncated form of the C3a (BC2 8.1 kDa). [75]. Mathelin et al. performed an independent validation study of BC1, BC2, and BC3 initially detected by Li et al. as 4.3 kDa, 8.1 kDa, and 8.9 kDa peptides [76]. This study failed to completely validate these biomarkers, but two peaks named BC1a (4286 Da) and BC1b (4302 Da) could be the same as Li's BC1 and BC2. Other peaks, such as BC3a (8919 Da) and BC3b (8961 Da) may be Li's BC3. BC1a and BC1b were significantly decreased, while BC3a and BC3b were

significantly increased in BrCa samples. Stringent cutoff values used as a panel detected 33 % of BrCas compared to 22 % detected by CA15.3 [77]. Another attempt to validate BC1, BC2, and BC3 performed poorly. BC2 had the highest diagnostic ability but did not achieve reasonable AUC to discriminate BrCa [78]. van Winden et al. also performed a validation study of BC1, BC2, and BC3. Here, four peaks at *m*/*z* 4276, 4292, 8129, and 8941 were detected and supposedly represent the previously reported proteins (4.3, 8.1, and 8.9 kDa) [79]. Only ITIH4 was confirmed and showed significant decreased expression in cancer samples. The *m*/*z* 8.1 kDa was not related to BrCa, and *m*/*z* 8.9 kDa was rather decreased in cancer samples compared to previous reported increases.

Quantitative validation study of already identified BrCa degradome including bradykinin, (des-Arg(9)-bradykinin), complement component 4a, and ITIH 4 using LC-MS/MS revealed median circulating concentrations of both ITIH4 and des-Arg(9)-bradykinin to be significantly higher in cancer than control samples. Importantly, the levels decreased to control range following surgical tumor removal. Both biomarkers were informative and contributed to cancer detection [80]. An absolute quantification of eight ITIH4 fragments (four between (658–687) and (667–687), the other four between (−30 and −21)) revealed high variability in concentrations between individual patients, but an overall trend toward increased levels in cancer patients was observed. Significant increases were observed for fragments 25 and 29.7, and these decreased after surgery [81].

A study focused on uncovering biomarkers for early detection of BrCas used pre-diagnosis serum samples from BrCa cohort in a prospective proteomic study. Samples from women diagnosed with BrCa 3 years after enrollment were compared to controls by SELDI MS. A subset had proteome identified and quantified by iTRAQ and 2D-nano-LC-MS/MS. Peaks at *m*/*z* 3323 and 8939 were significantly higher in pre-diagnosis BrCa sera. 2D-nano-LC-MS/MS revealed ITIH4, afamin, and apolipoprotein E to be significantly increased, while α-2-macroglobulin and ceruloplasmin were decreased in these samples [82]. A prognostic recurrent-free survival prediction by serum proteomics uncovered four peaks at *m*/*z* 3073, 3274 (ITIH4), 4405, and 7973 to have significant prognostic value. In multivariate Cox regression analysis, only *m*/*z* 3073 and 3274 (ITIH4), as well as 4405, retained their independent prognostic value in predicting recurrence-free survival [83].

Other Circulating BrCa Proteomic Biomarkers

In addition to (ITIH4), several other proteins and peptides have been identified as biomarkers of BrCa. Circulating BrCa early detection biomarkers have been targeted using SELDI MS, and candidate biomarkers were HPLC purified and LC-MS/MS identified and validated with protein chip immunoassay and Western blot. Three peaks identified as *m*/*z* 6630, 8139, and 8942 Da had a sensitivity of 96.5 % and a specificity of 94.9 % in blind testing. The 6630 Da peak was identified as apolipoprotein C-1 and was downregulated, the 8139 Da is the C-terminal truncated C3a, and the 8942 Da is the complement component C3a, and the latter two were upregulated. The levels of all three modulated with disease stage from

stage I to IV [84]. A 2-DE and MALDI MS study identified HSP27 to be upregulated and 14-3-3-σ downregulated in patient samples. The sensitivity and specificity of these biomarkers were 100 % and 97 %, respectively, for BrCa [85]. Seven biomarkers identified by SELDI MS in sera form HER2-positive BrCa patients could identify cancer patients from controls, achieving an AUROCC of 0.95. Sequence identity indicates a fragment of fibrinogen α (605–629) contributed most to the discriminatory ability, and the levels were lower in cancer patients but returned to normal postoperatively [86].

Kadowaki et al. originally described a three-step proteome analysis for low-abundant serum proteins and then applied this for discovery of biomarkers for DCIS. DCIS and control samples were subjected to immunodepletion, followed by reverse-phase HPLC and 2-DE. Western blotting and ELISA were used to confirm the biomarkers uncovered. Vitronectin was identified as significantly increased in DCIS, and an ELISA verified increased expression in DCIS and invasive cancer samples. Vitronectin is expressed by cells of small vessels surrounding cancer and by stromal cells [87].

Gast et al. applied a strict sample collection and processing procedure, coupled with SELDI MS to identify three serum and twenty-seven tissue peaks that were discriminatory [88]. Many peak intensities fluctuated (increasing or decreasing) with tumor progression from healthy to benign to progressive cancer stages. Two of the peaks in tissue samples were identified as N-terminal albumin fragments. A 2-DE approach, coupled with MALDI MS, was used to profile sera from patients with invasive ductal carcinoma and controls after depletion of high-abundant proteins. Six proteins (four isoforms of haptoglobin precursors and two of α1-antitrypsin (α1-AT)) showed differential expressions. Both biomarkers increased in samples from stage I to III patients. Alpha 1-AT is expressed in tumor tissue, with a tendency of increased expression in high-grade tumors [89]. Another cohort of plasma depleted of high-abundant proteins was subjected to ICAT labeling and tandem MS, and differentially expressed proteins were verified using immunoblot. Of four promising proteins, only biotinidase showed significant downregulation in BrCa samples in independent blinded samples, with an AUROCC of 0.78 [90]. To identify protein signatures predictive of metastasis in high-risk women on adjuvant chemotherapy, postoperative sera from high-risk early BrCa patients were analyzed by SELDI MS. A multiprotein index, inclusive of haptoglobin, C3a complement fraction, transferrin, apolipoprotein C1, and apolipoprotein A1, correctly predicted outcome in 83 % of patients. The 5-year metastasis-free survival was 83 % in those considered "good prognosis" compared to only 22 % for the "bad prognosis" category based on these protein signatures. This index retained its independent prognostic significance of relapse prediction in multivariate Cox regression analysis [91].

The potential of serum proteomics in uncovering BrCa biomarkers is evidenced by these numerous studies. Coordinated validation of some of these biomarkers is indicated.

4.4.7 Circulating BrCa Metabolomic Biomarkers

A large body of evidence confirms altered metabolites in primary BrCa tissue samples, in circulation, and in other body fluids of cancer patients. There are over 30 endogenous BrCa metabolites identified in tissue samples. The BrCa metabolome includes a spectrum of altered metabolites. Of interest are increased glycerophosphocholine, phosphocholine, and free choline. A magnetic resonance spectroscopic analysis of these metabolites show a broad single peak referred to as total choline-containing compounds. However, in vitro studies identify elevated phosphocholine as the major metabolite responsible for the peak. Indeed, an in vivo MRSI measurement of choline levels can yield a sensitivity of 100 % in detection of BrCa. Levels of phosphocholine also parallel disease stage as disease progression correlates with increases. Increased taurine, myoinositol, and phosphoethanolamine are also associated with BrCa. Consistent with other tumors, BrCa has low glucose levels as well.

Serum samples from BrCa patients have been analyzed mainly for use in prognostication, therapy prediction, and early detection of recurrences. The initial work by Asiago et al. used combined NMR and GC x GC MS to analyze the metabolome of 257 serum samples from 56 BrCa patients on surgical therapy to uncover signatures predictive of early relapse [92]. Multivariate analysis was used to analyze the data, and 11 informative metabolites were identified by logistic regression, and fivefold cross validation analysis. This validated study had a sensitivity of 86 % and specificity of 84 % (AUROCC 0.88) in detection of recurrences. An important finding was the ability to detect recurrences in 55 % of patients 13 months (on average) before conventional markers could detect them. Another study of metabolomic profile indicates the ability to identify ER-negative early BrCa patients at risk of relapse [93]. In an independent sample set, this study achieved a sensitivity of 82 %, specificity of 72 %, and a predictive accuracy of 75 % in determining the risk of relapse. Importantly, relapse was associated with significant increases in the levels of glucose ($p = 0.01$) and lipid ($p = 0.0003$) in association with decreases in histidine ($p = 0.0003$).

Oakman et al. used metabolomic profiling of pre- and post-operative sera to discriminate between early and metastatic BrCa [94]. This analysis performed at a sensitivity of 73 %, specificity of 69 %, and a predictive accuracy of 72 % in differentiating between the two groups. Of 21 patients determined to be at high risk for relapse, 10 preoperative samples from these patients were correctly predicted by metabolomic profiles. Similarly, of 23 patients at low risk of relapse, 11 preoperative and 20 postoperative metabolomic serum profiles were predictive. Using NMR-based metabolomic profiling, a sensitivity of 89.8 % and specificity of 79.3 % in an independent validation cohort were achieved in differentiating between women with metastatic BrCa from those with localized early disease. Importantly, this study identifies statistically significant differential levels of histidine, acetoacetate, glycerol, pyruvate, *N*-acetyl glycoproteins, mannose, glutamate, and phenylalanine. In women with metastatic BrCa, the outcome and response to

treatment was investigated by serum metabolomic profile. In a trial in which 579 patients were randomized to paclitaxel plus lapatinib (anti-HER2) or placebo, NMR spectroscopy was used to analyze pre- and on-treatment serial samples. This study uncovered a serum metabolomic profile that could predict women with HER2-positive tumors who respond to paclitaxel and lapatinib at a predictive accuracy for PFS of 89.6 % and OS of 78 % for the upper third and lower third of the data set [95].

Because urine is essentially a filtrate of blood, it serves as a convenient noninvasive sample for analysis of circulating metabolites. Thus, urine has been used to discover BrCa metabolomic biomarkers. The Kammerer's group has examined the utility of urinary ribonucleosides formed posttranscriptionally, for BrCa early detection. Using affinity chromatography and subsequent analysis with liquid chromatography–ion trap mass spectrometry on urine samples, modified ribonucleosides were able to differentiate between BrCa patients and controls. Thirty-one nucleosides achieved a sensitivity of 87.67 % and specificity of 89.90 % by support vector machine (SVM), which was more superior to the CA15-3 BrCa biomarker with a sensitivity of 60–70 % [96]. In a follow-up study, this group used 35 urinary candidate ribosylated nucleosides for classification of urine samples from BrCa patients and controls. This analysis achieved a sensitivity of 83.5 % and specificity of 90.6 % [97]. In another unique approach, gene expression profiles of tissue samples were used to uncover deregulated metabolic pathways in BrCa, and candidate molecules were tested on urine samples. Of nine altered metabolites, homovanillate, 4-hydroxyphenylacetate, 5-hydroxyindoleacetic acid, and urea achieved a consistent AUROCC of 0.75, 0.79, and 0.79 using linear discriminate analysis, random forest classifier, and SVM, respectively [98]. Urine samples from BrCa and ovarian cancer patients and healthy control subjects were subjected to NMR spectroscopy for differential metabolite detection. Using both univariate and multivariate analysis, unique metabolites were identified for BrCa and ovarian cancer, of which metabolites involved in the TCA cycle, amino acid, energy, and gut microbial metabolism were altered [99]. Thus, clinically useful BrCa metabolites can be measured noninvasively in circulation or urine.

4.4.8 *Circulating BrCa Cells*

Circulating BrCa cells (CBrCaCs) are established biomarkers for disease prognosis, treatment monitoring, and potentially useful as companion diagnostics.

4.4.8.1 Methods for Detection of CBrCaCs

Various methodologies have been used for CBrCaC enrichment, detection, and characterization. Circulating BrCa cells have mainly been enriched for, using density gradient centrifugation with separation of mononuclear cells from other

blood cells, as well as immunologic positive and negative selections. The FDA-approved CELLSEARCH® system has mostly been used for CBrCaC enumeration in patients with metastatic BrCa (MBC). Because of the absence of BrCa-specific surface antigens, CBrCaCs are generally defined as EpCAM-positive, CK-positive and CD45-negative cells. Additionally, morphologic criterion enhances phenotypic recognition. Molecular methods have also been used to target amplification of nonspecific epithelial markers such as *EPCAM* and *KRT*, as well as tissue-specific mRNAs such as *hMAM*, *HER2*, *ER*, and *PR*. Another comparable molecular assay is the Adna Test BrCa (AdnaGen AG, Langenhagen, Germany). This is a molecular assay whereby CTCs are first enriched for with immunomagnetic beads linked to anti-mucin 1 (MUC1) and EpCAM antibodies, followed by multiplex RT-PCR detection and quantification of gastrointestinal tumor-associated antigen (GA 733.2), EpCAM, and HER2. Other technologies can be used for CBrCaC characterization as well.

4.4.8.2 Prognostic Potential of CBrCaCs in Early Stage Disease

Occult spread of cancer cells, with formation of minimal residual disease, before clinical evidence of metastasis occurs in early BrCa, following curative intent surgery. These cells are responsible for recurrences even after complete surgical resection of early BrCa. The evidence for this is the detection of disseminated tumor cells (DTCs) in some patients at diagnosis or during surgery. The prognostic significance of micrometastatic BrCa, especially in early stage disease, was initially revealed by the study of DTCs in bone marrow. Bone marrow aspirates obtained at the time of surgery were stained with antibodies against epithelial membrane antigens and cytokeratins. Whereas studies in the 1990s uncovered a high frequency of DTC detection rate of ~30 %, possibly because of stage migration due to enhanced screening and surveillance, DTC detection frequency is now demonstrated to be as low as 3 % in early stage disease. Nevertheless their presence confers adverse outcome. Because the method of DTC collection is invasive, uncomfortable, and costly, efforts have been made in using CBrCaCs for prognostication and management of patients with early BrCa.

Several investigators have demonstrated the prognostic value of CBrCaC characterization in early BrCa patients on adjuvant therapy. While the detection rate is low and variable (CELLSEARCH® system detection rates is 9–55 %, RT-PCR detection rate is 2–30 %), the general consensus is that finding CBrCaCs in early BrCa is associated with decreased disease-free survival (DFS), BrCa-specific survival, and OS. For example, in the German SUCCESS trial that prospectively evaluated CBrCaCs in a large cohort of women with primary BrCa, the detection rate was 22 % by CELLSEARCH® system; however, the presence of $\geq$1 CBrCaC per 7.5 ml of blood at diagnosis before commencement of chemotherapy was an independent predictor of both DFS and OS [100]. Data on CBrCaC enumeration in locally advanced disease patients on neoadjuvant treatment is inconsistent but suggestive of equal prognostic relevance. In general, the prevalence of CBrCaC

detection during neoadjuvant treatment is low, but the prognostic value before, during, and after therapy needs to be established.

4.4.8.3 Prognostic Relevance of CBrCaCs in Metastatic Disease

Since the initial studies by Cristofanilli et al., several evaluations of the prognostic role of CBrCaCs enumeration using the CELLSEARCH® system have been reported. The system has a detection rate of 40–80 %. A number of clinical trials have assessed the prognostic relevance of CBrCaCs in the metastatic setting [101]. The pioneering study by Cristofanilli and colleagues was a prospective multi-institution double-blind study involving women with metastatic BrCa starting a new therapy regimen. Circulating BrCa cells were enumerated at baseline and at first follow-up visits after the initial therapy. Patients with ≥5 CBrCaCs per 7.5 ml of blood at both baseline and first follow-up had significantly worse PFS and OS. This study included two nested studies. One arm focused on the 47 % of patients in the study who were on first-line palliative treatment. Circulating BrCa cells were examined at baseline and on first follow-up visit. In this cohort, again median PFS and OS were significantly worse for those with persistently elevated CBrCaCs at both baseline and follow-up visits [102]. The other nested arm of this multi-institution study assessed CBrCaCs at subsequent follow-up visits for up to 9 months. The finding from this study offered confirmatory evidence that high CBrCaC levels predicts worse PFS and significantly dismal OS [103]. A single institutional retrospective study of metastatic BrCa patients who were either newly diagnosed or with recurrent disease, assessed CBrCaCs at time of diagnosis prior to the commencement of salvage treatment. Overall survival was significantly worse in patients with ≥5 CBrCaCs per 7.5 ml (median OS was 28.3 months vs. 15; $p < 0.001$; HR 3.64). This prognostic effect of CBrCaCs was independent of treatment choice, ER or HER2 status [104]. Another clinical trial found prognostic value of CBrCaCs in women with metastatic BrCa independent of hormone receptor or HER2 status of tumors or tumor location and number of metastatic deposits. Median PFS was 12 months for those with <5 CBrCaCs compared to 7 months for those with elevated CBrCaCs ($p < 0.001$). Even patients with ≥5 CBrCaCs at baseline demonstrated marginal survival benefit from first-line endocrine therapy despite positive hormone receptor status [105].

Giordano et al. examined CBrCaCs in a cohort of women with metastatic BrCa before the start of a new treatment [106]. Circulating BrCa cells correlated with histologic and phenotypic subtypes of cancer. Lobular histology and bone marrow metastasis were associated with high CBrCaCs. This is consistent with the findings of Dawood et al. that bone metastasis and elevated CBrCaCs conferred additional risk of death with HR of 1.61 [104]. It appears bone involvement is associated with increased CBrCaCs levels and poor survival. Additionally, HER2 targeted therapy was associated with decreases in CBrCaC numbers to <5 per 7.5 ml, even in patients with clinical and radiologic evidence of disease progression. In another prospective study, CBrCaCs decreased in patients on first-line chemotherapy in

addition to targeted therapy with trastuzumab or bevacizumab [107]. Wallwiener and colleagues found CBrCaCs to have strong independent prognosis in women with metastatic BrCa independent of molecular subtypes [108]. The reason for decreases in CBrCaCs even in patients with clinical evidence of disease progression is unclear, but might be due to selective elimination of HER2-positive CBrCaCs with little effect on the primary tumor or cells at metastatic sites.

4.4.8.4 Treatment Monitoring Using CBrCaCs in the Metastatic Setting

In metastatic BrCa, treatment response is evaluated by conventional imaging including improved FDG-PET and CT scanning. In view of this, CBrCaCs have been compared to these gold standards in disease monitoring. A number of studies suggest CBrCaC enumeration is superior to imaging in monitoring response to treatment in women with metastatic BrCa. A nested retrospective study compared the predictive potential of CBrCaCs to imaging in regard to response to treatment. In this cohort, the ~66 % of patients who showed radiographic response also had <5 CCBCs at the time of evaluation. Similarly, 16 % of those with progressive disease by imaging also had ≥ 5 CBrCaCs. This data, thus, shows concordance between imaging and CBrCaCs. However, response indicated by CBrCaC was significantly associated with improved median OS, irrespective of imaging evidence of response or nonresponse. Another study examined CBrCaC enumeration in comparison with FDG-PET/CT in women with metastatic BrCa. Both methods were measured at 3 months intervals after starting a new treatment for progressive disease. Although CBrCaC levels correlated with FDG-PET/CT evidence, CBrCaC enumeration was the most significant predictor of OS in this series. Increased CBrCaC counts during treatment suggested treatment failure and disease progression. A disadvantage that imaging suffers from, which is immune to CBrCaC enumeration, is intra- and inter-observer variability in image interpretation compared to CBrCaC counts (15.2 % vs. 0.7 %), making CBrCaC enumeration a more objective method for response monitoring. CBrCaC dynamics have proved useful in monitoring treatment efficacy in metastatic BrCa. However, the design of the SWOG So500 trial will offer further insight into the value of CBrCaC dynamics in treatment monitoring. One arm of this study examines the value of switching treatment (22 days after initial chemotherapy) in high-risk patients with persistently elevated CBrCaC counts (≥ 5 per 7.5 ml) despite chemotherapy.

4.4.8.5 CBrCaC Characterization Using Molecular Methods

Transcripts of a number of genes have been targeted as evidence of CBrCaCs. Their presence has been correlated with clinical outcomes in BrCa patients. Bonilla's group examined the expression of epithelial tumor markers, *KRT19* and *mammaglobin* (*hMAM*) in sera, and their relationship to clinicopathologic

parameters and to CBrCaCs. Sixty percent of BrCa patients were positive for *hMAM* and 49 % expressed *KRT19* compared with 12 % (*hMAM*) and 20 % (*KRT19*) in controls [109]. Both markers were associated with tumor size and proliferation index. Nine of ten patients with CBrCaCs had positive epithelial gene expression, but there was no statistical demonstration of this relationship. One can only surmise that CBrCaCs are associated with epithelial gene expression in this study. Another study of *hMAM* expression demonstrated that cell-free *hMAM* mRNA was a prognostic factor in women with BrCa [110]. Plasma *ERBB2* mRNA and CBrCaC characterization by *hMAM* transcript expression in the same women with BrCa was investigated. Forty-three percent had *ERBB2* mRNA in circulation, and this was independent of *ERBB2* expression in primary tumor tissues and was not associated with other clinicopathologic features of the disease. But *ERBB2* mRNA in plasma was significantly associated with negative ER and PR status of primary tumors. Plasma *ERBB2* mRNA also correlated with CBrCaCs, suggesting its association with aggressive disease [111]. A study by El-Attar et al. of *hMAM* mRNA in serum samples from BrCa patients aimed at its diagnostic use and possible detection of micrometastasis at initial diagnosis, alone or in conjunction with CA15.3 and CEA [112]. All the markers showed increased expression in BrCa compared to benign control group. CA15.3 and CEA levels significantly correlated with tumor size and grade. Plasma *hMAM* mRNA had the highest sensitivity and specificity at detection of both primary and metastasis BrCa. The use of the three markers in a panel enhanced the diagnostic accuracy for both primary (90 % sensitivity, 80 % specificity) and metastatic (100 % sensitivity, 79.2 % specificity) BrCa. Human *hMAM* expression in peripheral blood was compared to serum CEA and CA15.3 in patients with metastatic BrCa. CEA and CA15.3 were elevated in 51 % and 69 % of sera, respectively. Both as a panel achieved a sensitivity of 78 %. Human *hMAM* mRNA was positive in 54 % of cases. As an adjunct for detection of metastasis, the panel of *hMAM* mRNA and CEA was sensitive at 81 %, while *hMAM* and CA15.3 was 90 % [113]. *Maspin* and *hMAM* expression are specifically associated with BrCa. Hence Bitisik et al. examined their expression in peripheral blood as therapy response biomarkers [114]. This study suggested that both markers detected CBrCaCs and that *hMAM* mRNA determines efficacy of treatment, while *maspin* expression predicts aggressive disease.

4.4.8.6 Other Clinical Relevance of CBrCaCs

As part of the biology of metastasis, CBrCaCs interact with blood clotting factors such as fibrinogen, thrombin, tissue factor, and fibrin to form emboli. These cancer microemboli can be trapped in capillaries at distant metastatic sites that could extravasate and subsequently lead to the establishment of a metastatic deposit. In this regard, CBrCaCs have been related to thromboembolic phenomenon in women with metastatic BrCa. In patients with metastatic BrCa before commencement of palliative therapy, those with CBrCaCs of ≥ 1 per 7.5 ml of blood had fourfold risk

of developing thromboembolic events compared to patients without CBrCaCs. While there was a trend toward increase thrombosis in patients with ≥5 CBrCaCs, this finding failed to reach significance. Even when controlled for therapy and tumor burden in multivariate analysis, patients with ≥5 CBrCaCs still had elevated risk of thrombosis compared to those with no detectable CBrCaCs [115].

4.4.8.7 CBrCaCs as Companion Diagnostic Biomarkers

It is evident that the metastatic cascade is associated with emergence of clonal cell populations with different genetic and phenotypic features. HER2 and hormonal receptor status can change with disease progression and recurrence. Multiple studies have shown discordances in HER2 and hormone receptor expressions between primary tumors, CBrCaCs, and metastatic deposits. Fehm et al. in a large multicenter prospective study of primary tumors and CBrCaCs observed discrepancies in *HER2* expression between the two [116]. In another series, of the 66 % of patients with metastatic BrCa, 29 % with HER2-positive CBrCaCs had HER2-negative primary tumors, and similarly, 42 % with HER2-negative CBrCaCs had HER2-positive tumors [117]. HER2 amplification and overexpression are also demonstrated in CBrCaCs. There are similar discrepancies in endocrine receptor status, suggesting the need for real-time monitoring of tumor dynamics during therapy. The possible efficacious targeting of such evolved tumor clones has been demonstrated. Ten patients with persistent HER2-positive disseminated BrCa cells (DBrCaCs) were treated with trastuzumab for 12 months. On follow-up evaluations at regular intervals, it was finally observed that HER2-positive DBrCaCs were cleared in all patients [118]. Two clinical trials (DETECT and TREAT CTCs) are designed to test the clinical usefulness of CBrCaCs as surrogate endpoint biomarkers for the efficacy of targeting CBrCaCs in women with HER2-negative primary tumors. In the EORTC TREAT CTC phase II study (NCT01548677), patients with HER2-negative primary BrCa who have CBrCaCs are randomized to either receive trastuzumab or not (after neoadjuvant treatment). These clinical trials are aimed at treating HER2-positive CBrCaCs in women with primary HER2-negative tumors.

4.4.9 Circulating BrCa Stem Cells

BrCa stem cells (BrCaSCs) are defined phenotypically as $CD44^{+}/CD24^{negative/low}$. These cells have high tumorigenic potential and are able to self-renew and differentiate. Additionally, aldehyde dehydrogenase 1 (ALDH1), an enzyme involved in oxidation of aldehydes, is implicated in early differentiation of BrCaSCs and as such is being used as a marker as well. The ALDEFLUOR™ (STEMCELL Technologies) commercial assay targets ALDH1 to enrich for CBrCaSCs.

A large proportion of DBrCaCs have features of stem cells [119]. Whereas these cells constitute $<10\ \%$ of the primary tumors [120], Balic et al. found that up to 72 % of DBrCaCs had phenotypic characteristics of CBrCaSCs. Disseminated BrCa cells are found in the circulation as well. Stem cell features were present in 35 % of CBrCaCs [121]. The percentage of CBrCaSCs increased with increasing tumor stage [122]. BrCaSCs contribute to the clinical conundrum of failures in BrCa management:

- First, these cells are resistant to conventional chemotherapy and radiotherapy. Thus, disseminated BrCaSCs contribute to failures in adjuvant chemotherapy.
- Second, BrCaSCs among DTCs may be responsible for the ability to efficiently form metastatic deposits, and this partly explains why not all women with disseminated cancer cells suffer from relapse.

Flow cytometric analysis of bone marrow aspirates from BrCa patients concluded that women with high-risk clinicopathologic features harbored high levels of BrCaSC-like cells [123]. These findings suggest the need for further characterization of these cells to guide targeted therapy. This endeavor, however, posses a formidable challenge due to the paucity or rarity of CBrCaSCs and the possible presence of phenotypic subtypes for the various BrCa subtypes.

4.5 BrCa Extracellular Vesicles

In addition to being loaded with diagnostic biomarkers, BrCa-derived EVs (BrCaDEVs) are involved in cancer biology, from acquisition of invasive phenotypes, metastatic niche preparation, and treatment resistance, among other functions. The metastasis-inducing mR-200 family members can be transferred via extracellular vesicles from metastatic cancer to nonmetastatic cancer, to mediate EMT. These vehicles could induce metastasis in xenograft models [124]. Moreover, BrCaDEVs can induce invasive properties on other cells. This feature is partly mediated by the extracellular matrix metalloproteinase inducer (EMMPRIN) on these vesicles. Thus, anti-EMMPRIN strategies, such as deglycosylation, suppress transfer of invasive phenotypes by these vesicles. The p38/MAPK pathway was activated in recipient cells by these EMMPRIN-coated vesicles [125]. High levels of miR-122 in BrCaDEVs can suppress glucose uptake by non-tumoral cells in premetastatic niches by decreasing the expression of pyruvate kinase in receiving cells [126]. This helps increase available glucose to promote the glycolytic drive of BrCa cells.

4.6 Summary

- Primary BrCa presents as a conglomerate of different diseases that are histopathologically subclassified into numerous subtypes, albeit with some challenges. However, they are united by their origin, being from cells of the mammary gland.
- Molecular evidence recognizes only four categories of BrCa that have prognostic and predictive relevance.
- The molecular and genetic changes associated with primary BrCa are present in the circulation and breast tissue fluids, and have been demonstrated to have promising clinical applications.
- Proteomic approaches have uncovered a plethora of proteins/peptides with clinical potential.
- In fact, liquid biopsy applications of CBrCaC enrichment, enumeration, and characterization are in routine clinical practice, and this has prognostic, predictive, and companion diagnostic values.
- The cargo of BrCa-derived extracellular vesicles, including miRNAs, plays important roles in BrCa progression, as well as BrCaSC maintenance.

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Chapter 5
Esophageal Cancer Biomarkers in Circulation

Key Topics

- Risk factors of EsCa
- Screening for esophageal cancer (EsCa)
- Molecular pathology of EsCa
- Circulating EsCa biomarkers
- Circulating EsCa cells

Key Points

- EsCa remains a disease characterized by dismal outcomes. Geographically, esophageal adenocarcinomas (EACs) are more common in the resource-rich parts of the world, while those of squamous cell histology (ESCC) are more prevalent in middle- to low-income countries. The multistep EsCa progression model offers opportunity for noninvasive early detection; however, Barrett's esophagus is monitored by invasive endoscopic evaluations.
- Noninvasive biomarkers such as epigenetic alterations (e.g., promoter methylation of *CDKN2A*), miRNA deregulation (e.g., miR-18a), changes in protein levels (e.g., CYFRA 21-1), metabolite profiles (e.g., alterations in ketone bodies and glycolysis), and circulating EsCa cells should augment current efforts at disease detection and management.
- Circulating biomarkers are being pursued. However, while the molecular genetics of EAC progression is much better characterized, circulating biomarkers have been more extensively studied in ESCC.

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5.1 Introduction

Cancer of the esophagus has a major global presence. As the eighth and sixth cause of global cancer diagnosis and deaths, respectively, 456,000 new people were afflicted with 400,000 deaths in 2012. The 2016 estimated incidence and mortality are 16,910 and 15,690, respectively. Notice how the incidence closely mirrors the mortality, which is indicative of a cancer that has a high case fatality rate. Indeed, the 5-year survival is woefully 5–15 %. It is the third cause of gastrointestinal cancer mortalities. The global distribution of cases, which differs by 20-fold in order of magnitude between countries, reflects on the various etiologic risk factors. Seventy-five percent of all cases are diagnosed in Asia, with ~80 % of the global cases being clustered in the less developed world, where most of the mortalities also occur. Thus, attention to prevention, early diagnosis, and improved interventional measures need to be focused on this sector of the global population and not the currently otherwise observed.

There are two major pathologic subtypes of esophageal cancer (EsCa), esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). These two subtypes differ in their global distribution patterns, clinical and molecular features, as well as predisposing risk factors. The highest rate of EsCa, primarily ESCC, occurs in the less developed world, mainly in Eastern Asia, Southern and Eastern Africa, and what is described as the "esophageal cancer belt," which extends from Northern Iran through Central Asia into Northern and Central China. The lowest rates are observed in Central America and Western and Middle Africa. On the contrary, EAC is more common in the developed than the developing world. Obesity, which may be associated with gastrointestinal reflux disease (GERD) and Barrett's esophagus (BE), may help explain this prevalence. Lifestyle is a major contributor to EsCa, because in many Western countries, EAC is on the rise (mirroring the increase incidence of obesity), while ESCC is declining (due to declining tobacco use and moderate alcohol consumption). The reverse appears true for the developing world where smoking and alcohol use are on the rise, and hence the increasing incidence rates of EsCa.

As alluded to above, lifestyle plays a major role in the etiology of EsCa. Thus, to decrease the current rising trend, health educational and motivational efforts should be implemented to help people change the known risky habits. Complementing these efforts will be biomarkers that can play a key role in primary and secondary prevention. Moreover, while BE is a known risk factor for EAC, the majority of people with BE do not progress to develop invasive cancer and yet are subjected to the associated anxieties and importantly invasive endoscopic surveillance procedures. The clinical availability of validated biomarkers that can be used to stratify people with BE into "progressors" and "nonprogressors" will be invaluable in improving the current dismal outcomes of EsCa. Given that the majority (~80 %) of EsCas occur in the less industrialized world where endoscopy is not readily available, biomarkers that can be assayed noninvasively, especially in body fluids such as plasma, serum, and saliva, or otherwise, will make an even much impact on the global burden of this affliction. This chapter examines the molecular pathology

of EsCa and the current efforts at identifying and developing circulating biomarkers for this disease.

5.2 Risk Factors of EsCa

Although some overlap exists, such as tobacco and alcohol use, which may reflect on some of the identical molecular alterations, the risk factors for ESCC and EAC are quite different. Risk factors for both types of cancer include age (50+ years), thoracic radiation, and male sex (male:female ratio of 3:1 for ESCC and 8:1 for EAC). Familial predisposition is rare for ESCC but could be as high as 7 % for BE and EAC in European population.

Heavy alcohol consumption and smoking are established risk factors for ESCC. For alcohol use, the risk is further elevated especially in people with loss of activity of aldehyde dehydrogenase 2 (ALDH2). The glutamine polymorphic change to lysine at position 487 in *ALDH2* identified in Eastern Asians inactivates the protein. Drinking hot beverages or liquids is an identified risk factor for ESCC. This is one theoretical explanation for the increased ESCC in the "esophageal cancer belt." Achalasia (the incomplete relaxation of the lower esophageal sphincter on swallowing) is strongly associated with ESCC, while accidental caustic injury, especially during childhood, is associated with either ESCC or EAC. Other implicated risk factors for ESCC are fungal contamination of food, consumption of food poor in vegetables and fruits but high in nitrosamines, celiac disease, esophageal diverticula, and HPV 16 and 18 infections, as well as tylosis.

Chronic GERD that may lead to BE, increased body mass index (BMI), and smoking are proven risk factors for EAC. The frequency of GERD symptoms is associated with the risk of progressing to BE. In comparison to those who experience infrequent symptoms, weekly symptoms are associated with a fivefold risk, but this increases to sevenfold in if GERD symptoms occur daily. Smoking is associated with a relative risk of ~2.32 compared with nonsmokers, and BMI $\geq$ 35 confers a hazard ratio of 3.67 compared with those with normal BMI. Drugs such as β-blockers and anticholinergics that relax the gastroesophageal sphincter elevate the risk of EAC due to increased reflux.

5.3 Screening Recommendations for EsCa

The best approach to reducing the incident rates of EsCa is obviously prevention (avoidance of the lifestyle risk factors). Because the risk factors are different for ESCC and EAC, different interventional approaches are required. In the developed world, >90 % of ESCCs are caused by excessive alcohol and tobacco use. Conclusively, smoking cessation has reduced the incidence of ESCC in the developed world. GERD, especially in chronic situations, is associated with BE, both of which

are risk factors for development of EAC. However, the eradication of GERD and the associated reductions in the development of EAC are uncertain. But, as established risk factors, both GERD and BE warrant close monitoring for possible early detection of cancer.

In view of the grounded knowledge of chronic GERD and BE being risk factors for EAC, several organizations have recommended screening and surveillance procedures for people with these conditions. These organizations all agree on the recommendation that patients with chronic GERD and associated EsCa risk factors such as age ($\geq$50 years), male sex, white race, obesity, and the presence of hiatal hernia should have endoscopic evaluation of the upper gastrointestinal tract. The American Society for Gastrointestinal Endoscopy (ASGE) further refines these guidelines to include the following:

- Surveillance should stop in the event that there is no BE or EAC.
- In patients with non-dysplastic BE, surveillance intervals should be 3–5 years, and lesions should be biopsied for evaluation.
- Repeat biopsy in 6 months in patients with low-grade dysplasia (LGD) to confirm diagnosis and annually thereafter with biopsy of lesions. Consider lesion ablation at this time.
- In patients with high-grade dysplasia (HGD), endoscopic resection or radiofrequency ablation (RFA) of flat lesions is indicated. Surveillance should only be offered for those patients unfit or unwilling for surgery or RFA therapy.

To these surveillance and treatment guidelines by the ASGE, the American Gastroenterological Association (AGA) adds the following surveillance guidelines intervals:

- In the absence of dysplasia, repeat endoscopy in 3–5 years.
- In the presence of LGD, repeat endoscopy in 6–12 months.
- In the presence of HGD, for patients without eradication therapy, repeat endoscopy every 3 months.

The American College of Physicians issued in 2012 the following recommendations for endoscopic evaluation of patients with GERD:

- Endoscopic screening should not be offered to women of any age or men $<$50 regardless of risk factors, due to the low cancer incidence in these populations.
- Screening should be offered to both men and women with GERD, especially those associated with dysphagia, bleeding, anemia, weight loss, or recurrent vomiting.
- Surveillance should be stopped in those who have negative findings for BE on endoscopy.
- In patients with BE without dysplasia, surveillance should occur at 3–5-year intervals. For those with dysplastic lesions, surveillance intervals should be shorter.

Although there is evidence that some people can develop EAC without the classic metaplastic sequence through BE, there is no recommendation for

population-wide screening or at least for all men over the age of 50. Moreover, although >80 % of all EsCas occur in the resource-poor parts of world, where the subtype is primarily ESCC, there are no established recommendations for screening to detect early lesions of ESCC. This lack of generalized screening for the entire population is due partly to the disease prevalence and importantly the cost, invasive, and discomfort nature of endoscopy. Noninvasive body fluid biomarkers could make a difference, if developed and made available for screening of everyone above the risk age of 50.

5.4 A Place for Early Detection Biomarkers for EsCa

In patients with chronic GERD and/or BE, periodic endoscopic evaluation with detection and biopsy for grading of dysplasia is currently used to monitor risk for progression to EAC. Even with current improved endoscopic imaging techniques, including confocal laser endomicroscopy, narrow band imaging, and chromoendoscopy, there are still limitations to surveillance such as sampling errors or bias to this approach. The Seattle protocol attempts to provide gastroenterologists with a guide to comprehensive sampling, by recommending biopsy of all suspicious lesions, and four quadrant biopsy every 1–2 cm of the entire BE segment. While laudable, it has not been easily embraced, as complains surrounding this protocol stems from being time-consuming, tedious, and yet is still associated with sampling bias. Even if lesions are accurately sampled, histopathologic evaluation of biopsies are subject to the well-known inter-pathologist variable observations and opinions. Another issue with histopathologic evaluation and risk prediction is the fact that BE is not a boni fide index for developing EAC. While this invasive procedure is the recommended surveillance protocol for BE, the absolute risk for individuals to develop EAC following BE diagnosis is 1:200 per year (0.5 % or less per year). Indeed 90–95 % of patients with BE will not progress to EAC. The need to limit this invasive and costly procedure to BE patients at disproportionately high risk for progression is obviously laudable. However, endoscopy is not ubiquitous in the developing world where the majority of EsCas are diagnosed. Thus, an objectively measurable biomolecules (biomarkers) that can accurately predict the risk of lesion progression to either ESCC or EAC should complement histopathologic evaluations. Of even much better value and cost-effectiveness will be those biomarkers that can be evaluated in body fluids such as blood.

5.5 Molecular Pathology of EsCa

Cancers of the esophagus are anatomically mostly located in the distal two thirds. While ESCC is mostly found in the middle and lower thirds of the esophagus, EAC is mostly restricted distally close to the gastroesophageal junction (Fig. 5.1). In fact, it is often difficult to distinguish between cancers of the gastric cardia and EACs.

5.5.1 Barrett's Esophagus

Barrett's esophagus (BE), by definition, is the replacement of the stratified protective squamous epithelium of the lower esophagus with simple columnar epithelium. The metaplastic columnar epithelial cells are of three types, gastric-fundic type, cardia type, and intestinal type, that may contain goblet cells. Of the three, only the intestinal type of metaplasia carries a risk for progression and hence is the recommendation by the AGA and the American College of Gastroenterology for the diagnosis of BE. It is also established that noxious chemical substances including acid and bile from chronic GERD mediate the development of BE. The noxious

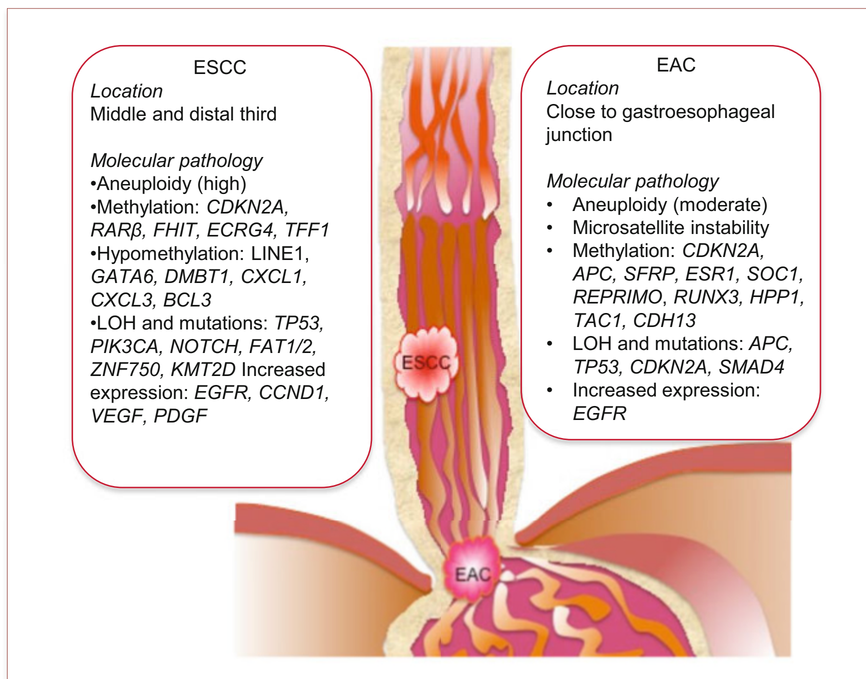

Fig. 5.1 Common anatomic locations and molecular pathology of esophageal adenocarcinoma (EAC) and squamous cell carcinoma (ESCC)

chemical damage is mostly repaired by replacement with normal stratified squamous epithelium. But in some individuals (probably those with increased frequency of GERD episodes or with different types of noxious substances that are yet to be determined), the epithelium trans-differentiates into the columnar type. The columnar epithelium is intended to be protective against reflux-mediated damage but may progress to invasive cancer. The metaplasia has been thought of as being a consequence of recommitment of the esophageal cells. However, other findings suggest different pathologic mechanisms. The cells that eventually differentiate into the columnar epithelia are suggested to be of three different "stem cell" origins: (i) the proximal migration of stem cells from the gastric cardia into damaged esophageal epithelium [1], (ii) the docking of circulating bone marrow stem cells at the damaged epithelial site, and (iii) the presence of embryonic stem cells located at the gastroesophageal junction [2].

The intestinal-type metaplastic change in the epithelium is an established predisposing risk factor for the development of EAC. In reference to only BE patients, the development of dysplasia, especially high-grade form, carries a risk of 10 % annually for progression to EAC. This figure contrasts sharply with the 0.2–0.5 % annual risk in patients without dysplasia. Globally, the incidence of EAC is on the rise, which suggests the ineffective detection of many people with BE and BE with dysplasia. To help curtail this trend, people with GERD, especially those with chronic and frequent symptoms, should be screened for early detection of BE that will inform actionable surveillance plan.

The need for objective measures to help stratify BE patients into risk categories is urgent. Biomarkers are available for assessing various lesions for predicting the risk of progression. Immunohistochemical stains for cyclin D1, p53, β-catenin, and α-methylacyl-CoA racemase (AMACR) are in clinical use. The established epigenetic and chromosomal changes in BE are also used as single or panel biomarkers to complement surveillance protocols. These biomarkers include aneuploidy and increasing tetraploidy, LOH at 9p and 17p, and methylation assays for targeting tumor suppressor genes such as *CDKN2A*.

5.5.2 Esophageal Cancer

ESCC is the most common pathologic type worldwide. It is more prevalent in males than females, and the median diagnostic age is 65. The numerous risk factors culminate in creating inflammation of the esophagus, the so-called chronic esophagitis. Squamous esophageal tumors are often located in the middle or lower third of the esophagus. Similar to other EsCas, presentation is often late, leading to poor survival outcomes.

Globally, EAC is the second most common cancer of the esophagus. These tumors are located at the lower esophagus (close to the gastroesophageal junction), because they arise on the background of BE, especially when it progresses to high-grade dysplasia. Similar to ESCC, men are more frequently affected than women.

Many patients present with late disease, whereby BE had not previously been diagnosed, and hence are often associated with poor prognosis. This is one of the rationales for the efforts to detect BE early, so that surveillance can be instituted for early detection of curable cancers.

5.5.3 Epigenetic Changes in EsCa

The epigenome is exploited early in EsCa progression. While these epigenetic alterations occur in both ESCC and EAC, they are better characterized in EAC. Evidence from BE, BE with dysplasia, and EAC conclusively demonstrates that these early epigenetic events in association with other genetic alterations drive EsCa progression.

5.5.3.1 Epigenetic Changes in EAC

Many genes, mainly tumor suppressor genes, are silenced through promoter hypermethylation in BE, BE with dysplasia, and EAC. While variable, many of the hypermethylation of these genes occur at a frequency of >50 % in EAC and at various reasonable frequencies in early lesions.

The tumor suppressor gene, *CDKN2A*, that encodes p16/INK4A is inactivated early in EAC development. This loss of function is due partly to promoter hypermethylation and LOH at 9p21, the *CDKN2A* locus. The early loss of this gene function in BE is associated with aneuploidy and LOH at 17p, the *TP53* locus. Together, these genetic changes drive clonal expansion of BE cells to invasive cancer. Other epigenetic alterations that may be associated with clonal expansion are promoter hypermethylation in *APC* and *ESR1* in BE and BE with dysplasia. Similarly, LOH and/or mutations in *APC*, *CDKN2A*, and *TP53* in early lesions may drive clonal progression. The methylation frequencies of *REPRIMO*, *SOC1*, and *SOC3* also suggest increased clonal expansion with disease progression. Promoter hypermethylation of *REPRIMO* is present in 36 % of BE cases but increases to 64 % and 63 % in BE with dysplasia and EAC, respectively [3]. Similarly, *SOC3* is methylated at a frequency of 13 % in BE, 22 % in BE with low-grade dysplasia, 69 % of BE with high-grade dysplasia, and 74 % of EAC. *SOC1* had similar progressive patterns of methylation, though at lower frequencies than *SOC3* [4].

Of major importance will be biomarkers that inform disease progression status. To address this question, Schulmann et al. identified inactivation via methylation of a number of genes associated with predictive increased risk of disease progression from BE to HGD or EAC [5]. These genes are *CDNK2A, HPP1,* and *RUNX3* with odd ratios of 1.74, 1.77, and 1.80, respectively. To improve disease stratification, the three methylated genes were combined with three other parameters, sex, BE segment length, and pathologic features, to generate ROC curves for prediction. For progression of BE to HGD or EAC, these ROC curves could accurately stratify

patients into low-, intermediate-, and high-risk categories [6]. To further augment the predictive accuracy of these biomarkers for disease progression, a multicenter retrospective study included five more relevant genes that are methylated in early disease, to create an eight-marker risk-of-progression panel. Using these eight gene panel (*CDKN2A, RUNX3, HPP1, TAC1, SST, NELL1, CDH13*, and *AKAP12*), when the specificity was set at 90 % using ROC curves, the predictive sensitivity was ~50 %. While not the best, this demonstrates an improvement and indicates that the selection of an appropriate panel could improve detection of disease progression.

5.5.3.2 Epigenetic Changes in ESCC

Several genes demonstrate promoter hypermethylation in ESCC. The frequencies though tend to be lower in low-, intermediate-, and high-grade dysplastic lesions than ESCC. Unlike EAC, the frequencies of gene promoter hypermethylation in ESCC, except for a few such as *CDKN2A, RARβ2, ECRG4*, and *FHIT*, are generally low.

Global methylation studies using genome-wide approaches have been applied to EsCa. Agarwal et al. demonstrated that BE patients who progressed to EAC were more likely than those who did not, to harbor hypomethylation in growth-promoting genes, including genes involved in insulin-like growth factor signaling [7]. Hypomethylation of long interspersed elements 1 (LINE1) is a frequent feature in ESCC, which correlates with poor prognosis [8]. In general, outside CpG islands, methylation is low in BE and EAC. Another study of genome-wide methylation, coupled with CGH, found that loss of methylation was an early event in disease progression and that global hypomethylation in association with gene amplification and increased expression of *GATA6, DMBT1, CXCL1*, and *CXCL3* may underlie disease progression [9].

5.5.4 Genetic Changes in EsCa

5.5.4.1 Chromosomal Alterations in EsCa

Chromosomal numerical (aneuploidy) and structural (LOH, chromosomal instability, amplification) changes are associated with EsCa. The frequency of aneuploidy is very high in ESCC, being present in up to 90 % of poorly differentiated tumors. Aneuploidy is also an early occurrence in EAC, and the frequency increases with lesion progression to invasive cancer. About 86 % of EACs harbors aneuploidy and may be associated with lymph node metastasis. The presence of these chromosomal numerical changes in BE was strongly associated with the risk of progression to HGD (69 % of “progressors” vs. 0 % of “nonprogressors”) [10].

LOH in several tumor suppressor genes and genes involved in DNA repair and cell cycle control occur in EsCa. Among these are losses at 2p21 (*MSH2* locus), 3p21 (*MLH1* locus), 5q21 (*APC* locus), 9q21 (*CNDN2A* and *CDKN2B* loci), 13q14 (*RB* locus), 17p13 (*TP53* locus), and 18q21 (*DCC* and *DOC4* loci). Similarly, growth factor genes, and genes involved in cell cycle progression, are amplified in EsCa. These include the following loci with involved genes; 7p12–13 (*EGFR* locus), 8q24 (*MYC* locus), 11q13 (*CCND1* locus), and 17q21 (*HER2* locus).

5.5.4.2 MSA in EsCa

Although not the predominant genetic alteration, MSI is associated with some EsCas. MSI often occur as a consequence of replicative error due to ineffective DNA mismatch repair, stemming from loss of function in repair genes including *MLH1* and *MSH2*, which are altered in EsCa. The frequency of MSI is higher in EAC than ESCC. In EAC, 10–20 % of cases demonstrate MSI. While variable, a good proportion of high-grade dysplastic lesions of EAC (~33 %) and ESCC (~23 %) samples harbors *MLH1* promoter hypermethylation, implicating them in possible MSI.

5.5.4.3 Alterations in Cell Cycle Regulators in EsCa

RB loss of function is common in both ESCC (30–50 %) and EAC (35–50 %). The primary mechanism of *RB* inactivation is LOH. In ESCC, this appears to be a late event that occurs on a background of increased expression of *CCND1*, loss of *TP53*, and decreased expression of *CDKN2A*. However, in EAC, *RB* loss of function is an early occurrence, with increasing rates of loss in dysplastic lesions and carcinoma.

TP53 LOH and mutations are common in both major types of EsCa. In ESCC, *TP53* mutations occur in up to 93 % of cases (range 40–75 %) and have been observed in early mucosal lesions. The frequency of these mutations increases in a tumor-progressive fashion, from hyperplasia to dysplasia to carcinoma. As an early event, *TP53* mutations or LOH precede *RB* loss of function. The types of *TP53* mutations appear as a fingerprint of the causative agent. Of interest, most *TP53* mutations commonly occur in the DNA-binding domain encoded by exons 5–8. In about 40 % of ESCC, *TP53* mutations are A/T base pairs, which are changes that can be induced by a metabolite of ethanol, acetaldehyde. Additionally, 15 % of cancers harbors G to T transversion mutations, which are reminiscent of tobacco-related exposure. In 18 % of cases, C to T transition mutations are observed.

TP53 loss occurs in 45–75 % of EACs. Similar to ESCC, these mutations mostly involve exons 5–8, and are mainly base transitions. *TP53* mutations also demonstrate clonal expansion in the epithelium and mirror the progressive metaplasia-to-dysplasia-to-carcinoma sequence. The mutation rate ranges from ~5 % in BE to 65–75 % in dysplastic lesions and to 90 % in EAC. In the progressive sequence, *TP53* mutations precede and may contribute to the development of aneuploidy,

because diploid dysplastic cells with *TP53* mutations arrest at G2 phase and undergo aberrant replication that can give rise to aneuploidy. While *TP53* lesions coexist with those of *CDKN2A*, it is evident that 9p LOH (*CDKN2A* locus) occurs prior to 17p (*TP53* locus), and these early events precede LOH at 5q, 13q, and 18q.

The gene products from the 9p21 locus, p16 and p15, inhibit cyclin D1:CDK4/6 complexes to promote cell cycle arrest at G1. The 9p21 locus is frequently lost in dysplastic lesions (90 % frequency) and EAC (80 %). Although inactivation of *CDKN2A* by mutation, primarily in exons 1 and 2, occurs in ~25 % of cases, promoter hypermethylation is more common and is very frequent in dysplasia and adenocarcinoma. Similarly, loss of *CDKN2A* via promoter hypermethylation is found in ~50 % of ESCC. Genetic defects of *CDKN2B* in EsCa are less well documented. LOH at 9p also precedes losses at 5q and 13q.

Overexpression of *CCND1* (11q13) due to gene amplification occurs in 30 % of BE or dysplastic lesions but in as many as 70 % of ESCC. About 92 % of EACs overexpress *CCND1*, but gene amplification is rare, suggesting alternative mechanisms that are less well understood. The overexpression of *CCND1* is of prognostic significance, being associated with metastasis, advanced tumor grade and stage, poor chemotherapy response, and decreased overall survival. Cyclin E is also overexpressed in BE with dysplasia, EAC, and ESCC.

5.5.4.4 Growth Factor Alterations in EsCa

Exogenous and endogenous growth ligands can propel cell cycle uncontrollably by inducing cyclin expression. Tyrosine kinase receptors of importance in EsCa are the EGFR and FGFR-α. Also elevated in EsCa are VEGF and PDGF, which are associated with angiogenesis.

While growth factors may not be overexpressed in ESCC, the amplification and overexpression of their receptors are well documented in ESCC. *EGFR* (c-erb B1) is overexpressed in 40–70 % ESCCs and may predict poor chemotherapy response and adverse outcome. Both *EGFR* and *TGFα* are overexpressed in BE and EAC, and *EGFR* overexpression correlates with lymph node metastasis.

EGFR2/*HER2* (c-erb B2) is overexpressed in both EAC (~20 %) and ESCC (~10 %). The mechanism of elevated expression is gene amplification. Whereas its clinicopathologic relevance in ESCC is less understood, *HER2* overexpression in EAC predicts disease progression and correlates with aneuploidy. The elevated levels of HER2 are strong independent prognostic factors of EAC.

Angiogenesis is a hallmark of cancer, necessary for sustained growth, invasion, and metastasis. *VEGF* and *VEGFR* overexpression are detected in BE. *VEGF* overexpression can be observed in ~58 % of ESCC and is an early event in EAC, being present in metaplastic lesions. *PDGF* overexpression in ESCC is associated with increased VEGF levels, increased microvasculature, venous invasion, and poor survival.

5.5.4.5 Apoptosis and Immune Escape in EsCa

The ability to avoid death by any mechanism is another hallmark of all cancer cells. For EsCas, this is achieved through multiple mechanisms, as summarized below:

- EsCa cells alter the expression of death receptors. The interaction of the surface receptor FAS, with its ligand FAS-L, leads to cellular death via apoptosis. Normal cells express FAS; however, both ESCC and EAC cells lose surface expression of FAS, so as to avoid apoptotic cell death. Both ESCC and EAC and their precursor lesions overexpress FAS-L. Thus, these cells avoid apoptotic cell death by decreasing expression of FAS but are able to kill FAS-expressing cells such as immune surveillance lymphocytes through their interactions with FAS-L on cancer cells. Expression of FAS-L is thus associated with decreased tumor-infiltrating lymphocytes. Thus, tumors that express FAS receptor confer independent prognosis of prolonged disease-free survival.
- The loss of p53 functions, as is common in EsCa, prevents activation of pro-apoptotic genes in situations of irreparable DNA damage.
- EsCa cells increase expression of Cox2 enzyme, which is an inhibitor of apoptosis. Cox2 is overexpressed in BE and shows progressive increases in levels in dysplastic lesions and carcinoma cells.

5.5.4.6 Adhesion Molecules and Invasion of EsCa Cells

The two adhesion molecules of importance in EsCa are E-cadherin and β-catenin. E-cadherin is important in cell–cell adhesion and is anchored to the cell cytoskeleton via α-, β-, and γ-catenins. Loss of E-cadherin thus causes cellular detachment and invasion. β-catenin also serves adhesive functions, but free unantagonized β-catenin can translocate into the nucleus to trigger signal transduction that enhances cellular proliferation, growth, and antiapoptosis. Expectedly, therefore, E-cadherin expression is lost or decreased in 45–80 % of ESCC, with decreasing levels being associated with degree of metastasis. The prognostic value of E-cadherin has been proven. Low levels are associated with worse outcome variables such as increased hematogenous spread and mortality. Loss of E-cadherin is associated with poor 5-year survival outcome. The progression of BE to EAC is also associated with decreased E-cadherin expression. In 65 % of EAC, E-cadherin expression is reduced via LOH at 16q22, the *CDH1* locus. In both ESCC and EAC, β-catenin changes from membrane to become cytoplasmic with disease progression.

5.5.4.7 Other Alterations in EsCa

APC tumor suppressor gene inactivation via LOH occurs in about 55–80 % of ESCC and in 20–55 % of EAC. Mutations are infrequent in EAC, occurring in

< 10 % of cases. However, loss of *APC* is a late event in EsCa. Cancer cells avoid telomere shortening that halts the cell cycle, by increasing the expression of telomerase. Telomerase helps stabilize the lengths of telomeres in these cancer cells. Almost all (100 %) EsCas (ESCC, EAC, BE with dysplasia) overexpress telomerase.

5.5.5 *Multistep Esophageal Carcinogenesis*

The multistep carcinogenic pathway is well charted for EsCa. Histopathologic, molecular, and genetic characterization of tumors has enabled two different pathways for EAC and ESCC progression. EAC progression employs the metaplasia–dysplasia–adenocarcinoma sequence, while the hyperplasia–dysplasia–squamous cell carcinoma sequence characterizes ESCC progression.

The progression of BE through LGD, HGD, and eventually EAC requires epigenetic and genetic changes with clonal selection and development of expansive preconditioned cancer fields. Subsequent additional genetic alterations usher in the invasive pathology. Myriads of molecular genetic lesions occur to drive EAC progression, but a number of genetic changes have been well placed in the progressive sequence. Hypermethylation of *CDKN2A* and loss of cell cycle control, followed by loss of *TP53* through mutations that may contribute to G2 arrest, abnormal replication, and hence, subsequent aneuploidy, underlie development of BE and eventual dysplastic lesions. Loss of *RB* tumor suppressor functions adds to other cell cycle deregulation. Overexpression of *HER2* further confers in these cells the ability of growth independent of exogenous factors. Additional factors are mutations in *SMAD4* in HGD and EAC.

TP53 mutations (distinct from those in EAC) occur early in ESCC and may be involved in cell cycle deregulation and genomic instability. Augmenting this, and to offer the cell with uncontrollable proliferation, is *CCND1* overexpression. Self-sufficiency in growth signals is partly acquired via *EGFR* overexpression. However, acquisition of invasive and metastatic phenotype is through loss of *CDH1* expression and alterations in β-catenin levels.

5.6 Circulating EsCa Biomarkers

Circulating cell-free DNA has diagnostic potential in EsCa; however, targeting tumor-specific genomes through detection of epigenetic and genetic alterations may be more clinically useful. Changes in coding and noncoding transcripts, serum proteins, and metabolites in circulation of EsCa patients have been demonstrated. The potential utility of circulating EsCa cells in disease management is worthy of validation.

5.6.1 Circulating Cell-Free Nucleic Acid Content as EsCa Biomarkers

The clinical relevance of ccfDNA has been addressed in EsCa patients. Esophageal and gastric cancer patient plasma samples were collected preoperatively for diagnostic assessment. Short (102 bp) and long (253 bp) DNA amplicons and DNA concentration were measured. DNA concentrations of both short and long fragments were much significantly elevated in cancer patients compared to controls. This difference was more pronounced in EsCa patient samples. The AUROCC was 0.83 and 0.91 for short and long fragments, respectively, for EsCa, and 0.75 and 0.67 for short and long fragments for the gastric cancer cohorts. DNA integrity index defined as the ratio of short to long fragments, significantly differentiated EsCa patients from normal healthy controls [11]. Other studies support the elevated levels of ccfDNA in patients with EsCa. Banki et al. found that plasma DNA was more reliable than CEA for the detection of recurrences [12]. They concluded that elevated ccfDNA was significantly higher in cancer patients than controls but that these high levels returned to normal following complete surgical resection. Similar to other cancers, EsCa is associated with increased ccfDNA.

5.6.2 Circulating EsCa Epigenetic Biomarkers

Because of the importance of epigenetic alterations in EsCa progression, attempts have been made to uncover their clinical relevance in circulation. *APC*, *CDKN2A, TAC1, MSH2,* and global methylation changes in circulation are associated with EsCa.

Circulating methylated *APC* has been assayed as a prognostic biomarker of EsCa. *APC* (5p21–22) is frequently silenced via methylation in EsCa, and this has been investigated in plasma samples as a prognostic biomarker. Tumor and matched plasma samples were tested, and hypermethylation was in 92 % of EAC, 50 % of ESCC, and 39.5 % BE. The detection rates in plasma samples were 25 % and 6.3 % for EAC and ESCC, respectively. However, high circulating levels were significantly associated with reduced survival [13]. Hoffmann and colleagues also targeted methylation of *APC* and *DAPK* in plasma samples from patients with EsCa [14]. Methylation of either gene was found in 61 % of cancer patients. Preoperative levels of methylated *APC* and *DAPK* predicted poor survival outcomes. Both biomarkers significantly increased the accuracy of discriminating between short-term (<2.5 years) and long-term survival, and postoperative detection of methylated *APC* promoter predicted residual tumor presence.

Given the early promoter methylation of *CDKN2A* in EsCa progression, it has been explored as diagnostic biomarker. Promoter hypermethylation of *CDKN2A, CDH1,* and *RARβ* assayed in blood was compared to the expression of CEA (marker of CTCs) for the detection of EsCa. At least one gene was methylated in

37 % of the samples. CTCs (*CEA* expression) were detected in 37 % of samples as well. Methylation had no correlation with CEA expression, which suggests complementary utility for EsCa detection [15]. In a follow-up study, CTCs were detected at a rate of 27 %, and methylation of at least one of the three genes was in 37 % of the samples. The combined application of the four biomarkers enabled the detection of 53 % of EsCa patients. This assay was specific, because all control samples were negative [16]. In an earlier study, Hibi et al. detected *CDKN2A* promoter methylation in as many as 82 % of serum samples from EsCa patients [17].

Other genes assayed in circulation of EsCa patients are *TAC1* and *MSH2*. *TAC1* promoter methylation was analyzed in 258 EsCa samples at various stages of disease progression and 126 plasma samples. Gene methylation status could significantly distinguish cancer from normal tissues ($p < 0.0001$), and the frequency of *TAC1* methylation increased with disease progression from BE (55.6 %), BE with dysplasia (57.5 %), to EAC (61.2 %). Moreover, *TAC1* methylation was associated with BE segment, which is a clinical measure of risk for progression. Mean normalized methylation values and frequencies in plasma were significantly higher in EsCa patients than controls. *TAC1* was methylated in 50 % of ESCC tissue samples as well, and this was associated with poor OS [18]. The promoter methylation of *MSH2* in plasma samples was used to monitor disease-free survival after esophagectomy in ESCC patients. In this cohort, methylation was found in 48.3 % of matched tissue and plasma samples, of which 76.2 % harbored same methylation in both sample types. Postoperative DFS was lower in patients with high *MSH2* methylation compared to those without [19].

A methylome approach was explored by Zhai et al., who used the Infinium HumanMethylation 27 BeadChip that covers 27,578 CpG loci in 14,495 genes to interrogate tissue and matched sera from EAC, BE patients, and healthy controls [20]. In cancer patients who provided both tissue and serum samples, there was a strong correlation ($r = 0.92$) in methylation patterns between the two. Using the most differentially methylated loci for hierarchical clustering, 911 loci could perfectly separate EAC patients from controls, 554 loci distinguished between BE and EAC, and finally 46 loci discriminated between BE and controls.

5.6.3 Circulating EsCa Genetic Biomarkers

Genetic alterations as circulating biomarkers of EsCa have been assayed. In both tumor and matched serum samples from patients with ESCC, 92.9 % had at least one MSA in primary tumor tissue samples, when 12 markers located at 5q (*APC*), 9p (*CDKN2A*), 17p (*TP53*), and 18q (*SMAD4*) were used. Interestingly, 96.4 % of the positive cases also had at least one alteration in matched serum samples. Controls were all negative [21]. Using the same markers in a follow-up study, the detection frequencies were 84.4 % and 81.3 %, respectively, for tissue and serum samples. As an early detection biomarker, all early stage disease patients (no lymph

node involvement, pT1pN0) harbored serum MSA with none in control subjects [22]. Similar findings were further uncovered in serum and tissue samples. In this cohort of patients with EsCa of distal esophagus and gastric cardia, LOH in tissue and serum samples ranged from 77 to 96 %, and similarly with none in control serum samples [23].

5.6.4 Circulating EsCa Coding RNA Biomarkers

In EsCa, several gene transcripts have been assayed in circulation as biomarkers of disease progression, prognosis, and possible monitoring for treatment efficacy. *SCCA2* mRNA in circulation as a biomarker for ESCC progression from hyperplasia to dysplasia and finally to invasive cancer has been examined. *SCCA2* mRNA and protein levels in circulation were both measured. Also ESCC tissue samples were tested. Normalized *SCCA2* positive rates were 82 %, 60 %, 48 %, and 36 % in cancer, dysplasia, hyperplasia, and control groups, respectively. The difference between cancer and the rest was significant, so was the difference between dysplasia and control groups. *SCCA2* mRNA in blood was 97.5 % concordant with tissue expression, which also correlated with serum protein levels [24]. Another study from this same group examined the expression of *hTERT* and *EYA4* in blood as a biomarker of ESCC progression [25]. The expression of both genes increased with disease progression from hyperplasia to dysplasia and to cancer. The inclusion of both markers with traditional risk factors increased the risk-stratification accuracy. The sensitivity and specificity of *EYA4* were 70 % and 76 %, respectively, while *hTERT* achieved a sensitivity of 80 % and a specificity of 88 % for ESCC detection. This assay appears valuable for selecting patients for a more invasive endoscopic evaluation and also for monitoring disease progression from dysplasia to cancer.

The potential of circulating transcripts as prognostic biomarkers of EsCa has also been examined. In a critical care setting where patients received surgery, preoperative serum mRNA levels of 11 inflammation-related genes were assessed and monitored chronologically postoperatively for a couple of weeks. In multivariate analysis, increased intraoperative *IL-6*, *vWF*, *TGFβ1*, postoperative *MUC1*, and *NAMPT* mRNA levels were significant independent factors of death in the first year [26]. In another cohort of EsCa patients undergoing surgery, pre- and post-operative circulating *KRT19* mRNA (this probably detected CTCs) was associated with various clinicopathologic features. Patients with lymph node metastasis, relapse, and distant metastasis had increased circulating *KRT19* mRNA levels, and shorter DFS was associated with only high postoperative levels. Shorter DFS was predictable with high preoperative levels in patients with stage III metastatic ESCC. Expectedly, decreasing *KRT19* transcript levels postoperatively was associated with good prognosis [27]. The value of circulating *SCCA* transcripts as biomarker of disease relapse in patients who received curative-intent esophagectomy for ESCC was explored. Patients with high levels of the transcripts had higher probability (71 %) of tumor recurrence at 2 years earlier than those with lower levels

(27 %). In multivariate analysis, *SCCA* mRNA was the strongest independent predictor of recurrence [28].

Circulating gene transcripts as treatment prediction biomarkers have also been examined. Whole genome expression analysis of blood samples from ESCC patients before and after chemoradiotherapy revealed a predictive value of *FAM84B* mRNA levels. *FAM84B* is overexpressed in ESCC tissue and cell lines. Upon neoadjuvant chemoradiation treatment, decreasing serum levels of *FAM84B* mRNA and protein was predictive of pathologic complete response, and was thus associated with favorable outcome [29].

5.6.5 Circulating EsCa Noncoding RNA Biomarkers

Esophageal carcinogenesis is partly controlled by aberrant expression of ncRNAs. Well-characterized deregulated ncRNAs are miRNAs and to a lesser extent lncRNAs. Both classes of ncRNA control cancer cell proliferation, growth, differentiation, apoptosis, and other hallmarks of cancer. They have thus been assessed as biomarkers for screening, diagnosis, and prognosis and as treatment targets. While extensive data exist on their tissue expressions and functions, the aberrant levels of these ncRNAs in circulation have also been explored and have shown translational potential.

5.6.5.1 Circulating EsCa MiRNA Biomarkers

The cancer biology of miRNA in EsCa uncovers numerous relevant targets. Overexpressed oncomirs and their targets in EsCa are miR-233 (*FBXW7*), miR-21 (*PTEN, PDCD4, BCL2, TMP1, maspin*), miR-183 (*PDCD4*), miR-106 (*KLT4*), miR-328 (*GNG7*), miR-9, miR-25 and miR-92a (*CDH1*), miR-16 and miR-208 (*SOX6*), and miR-373 (*LATS2*). Similarly, tumor suppressormirs and their targets in EsCa include miR-203 (*TP53, BMI1, LASP1*), miR-29c (*CCND, CCNE*), miR-27a (*KRAS*), miR-302b and miR-520a (*ERBB4*), miR-375 (*IGF1R*), miR-133a (*CD47, MMP1, FSCN1*), miR-593* (*PLK1*), miR-326 (*VEGFC*), miR-195 (*CDC42*), miR-625 (*SOX*), miR-100 (*mTOR*), miR-200 and miR-205 (*ZEB*), and let-7 family (*RAS*). There is a complex regulatory network of miRNAs and their target genes in EsCa. The circulating levels have been explored as diagnostic and prognostic biomarkers.

Circulating EsCa Diagnostic miRNA Biomarkers

The deregulation of miRNAs in circulation of EsCa patients has previously been demonstrated; however, Zhang et al. first revealed the clinical usefulness of circulating miRNA in ESCC [30]. They uncovered seven miRNAs in serum with potential for detection of ESCC. This miRNA panel achieved a sensitivity of 78.5 %, a specificity of 96 %, and an AUROCC of 0.929. As single biomarkers,

miR-22 was the best performer with sensitivity of 88.6, specificity of 86.0, and AUROCC of 0.949. Importantly miR-22 was elevated in early stage disease. The lowest performer, miR-100, even achieved an AUROCC of 0.817.

The levels of plasma miR-21 were much higher, while miR-375 was significantly lower in ESCC patients than controls. As expected, the ratio of miR-21 to miR-375 was significantly higher in patients than controls. The diagnostic AUROCC was 0.816 in validation studies. The high miRNA ratio was associated with vascular invasion and disease recurrence [31]. Circulating levels of miR-16, miR-21, miR-185, and miR-375 were significantly higher in ESCC patients than controls. The elevated miR-375 in this cohort is unclear; however, as a single biomarker, mR-375 alone had AUROCC of 0.925 [32]. The levels of miR-21, miR-100, miR-193-3p, miR-194, miR-223, miR-337-5p, and miR-483-5p were significantly higher in ESCC patients than controls. As a panel, they achieved an AUROCC of 0.83. The elevated circulating levels declined after surgery [33].

Another miRNA with diagnostic potential in EsCa is miR-18a (from miR-17-92 cluster) that is highly expressed in cancer tissue samples. The significantly elevated expression of miR-18a in ESCC tissues and cell lines reflected in plasma levels ($p = 0.001$), and the circulating levels were reduced postoperatively. As a diagnostic biomarker, the AUROCC was 0.9449 for all patients, 0.9479 for stage pTis-I, and 0.9642 for pStage 0-I patients [34]. In a follow-up study by Komatsu et al., miR-18a was highly expressed in a number of cancers including esophageal, pancreatic, and colorectal cancers and hepatocellular carcinoma. Serum or plasma levels of miR-18a achieved a diagnostic AUROCC of 0.944 for EsCa [35].

Zhang et al. demonstrated the diagnostic role of miR-31 in ESCC. Levels of miR-31 are increased in tissue and serum samples from ESCC patients. The serum levels achieved diagnostic accuracies with AUROCC of 0.902 and 0.888 for the training and validation data sets, respectively [36]. Another promising circulating ESCC miRNA is miR-1322 that reached a diagnostic sensitivity of 81.7 %, specificity of 82.5 %, and AUROCC of 0.847.

There are several other potential useful diagnostic miRNAs for ESCC. An array-based analysis of serum samples uncovered markedly elevated miR-1246 in ESCC patients. This miRNA levels achieved diagnostic sensitivity, specificity, and AUROCC of 71.3 %, 73.9 %, and 0.754, respectively [37]. In serum samples, the levels of miR-29c and miR-205 were significantly more decreased, while those of miR-10b appeared much higher in ESCC patient samples than controls. As noninvasive diagnostic tool, miR-10b, miR-29c, and miR-205 achieved AUROCC of 0.85, 0.72, and 0.72, respectively [38]. Plasma let-7 and miR-20a have diagnostic potential in patients with ESCC. Circulating levels of let-7 are lower and miR-20a are higher in ESCC patients. The optimal diagnostic AUROCC was 0.829 (sensitivity of 74.3 %, specificity of 85 %) for let-7 and 0.767 for miR-20a (sensitivity of 64.3 %, specificity of 75 %) [39]. Hui et al. identified miR-365 as ESCC diagnostic biomarker based on multivariate logistic regression analysis [40]. This serum miRNA achieved a sensitivity, specificity, and AUROCC of 80.6 %, 86.7 %, and 0.831, respectively. Of interest, this study also uncovered miR-129 to differ significantly with disease stages (I/II vs. III and III vs. IV). Circulating levels of miR-155

and miR-183 were significantly reduced in EsCa patient samples compared to controls. When adjusted for tobacco and alcohol use, miR-155 was significantly associated with increased risk for EsCa and, as a diagnostic biomarker, achieved an AUROCC of 0.66 [41].

Circulating EsCa Prognostic miRNA Biomarkers
MiR-21, miR-31, miR-200c, miR-375, and miR-1246 among others appear to relate to prognostic variables in EsCa patients. High serum levels of miR-31 were an independent marker of adverse relapse-free survival (HR 3.26) [36]. Similarly, high plasma miR-21 levels are associated with vascular invasion, tumor recurrence, and poor 3-year survival. But high plasma miR-375 levels are associated with better survival outcomes. Multivariate analysis indicated high miR-21, and low miR-375 is an independent prognostic predictor of poor survival [42]. The serum levels of miR-21, miR-145, miR-200c, and let-7c were significantly higher in EsCa patients than controls. However, only miR-200c levels were significantly associated with poor response to chemotherapy and shortened PFS. Multivariate analysis confirmed the prognostic value of elevated serum miR-200c in EsCa patients on neoadjuvant chemotherapy [43]. Both miR-16 and miR-21 are elevated in EsCa patients, and the elevated circulating levels of miR-16 were associated with advanced stage disease, and by Kaplan–Meier survival analysis, both plasma miR-16 and miR-21 were significantly associated with shortened PFS and OS [32]. The prognostic utility of miR-21 and miR-375 in EsCa has been demonstrated in meta-analysis of 21 studies involving 2258 people. Of 39 miRNAs associated with prognosis, the pooled hazard ratio of miR-21 expression as a significant predictor of OS in ESCC was 1.84. That for miR-375 was also significant at 0.55, suggesting high miR-21 and low miR-375 conferred poor OS [44].

The levels of miR-21, miR-100, miR-193-3p, miR-194, miR-223, miR-337-5p, and miR-483-5p were significantly higher in ESCC patients than controls. High miR-25 levels correlated with shorter OS. In Cox regression analysis, lymph node metastasis, miR-25, and miR-100 levels were of value as independent predictors of survival (HR of 2.98 for LN metastasis, 3.84 for miR-25, and 4.18 for miR-100) [33]. MiR-613 levels are much reduced in serum samples from ESCC patients. The AUROCC for all patients and early stage disease patients were 0.767 and 0.728, respectively. By multivariate and Kaplan–Meier analyses, decreased miR-613 levels were an independent predictor of OS and PFS [45]. As a prognostic biomarker, miR-1246 was significantly associated with tumor stage and was a strong independent predictor of poor OS (HR 4.032) [37].

5.6.5.2 Circulating EsCa LncRNA Biomarkers

A number of lncRNAs are aberrantly expressed in EsCa tissue compared to normal control samples, and some have been linked to clinicopathologic parameters. Several studies have been on ESCC tissue samples. Many lncRNAs are overexpressed and associated with advanced disease features. Specifically,

HOTAIR, MALAT1, SPRY4-1T1, UCA1, PEG1O, PlncRNA1, FOXCUT, and its interacting partner *FOXC* are all overexpressed in ESCC tissue samples. These elevated levels are associated with advanced tumor stage, increased invasiveness, lymph node and distant metastasis, and poorly differentiated tumors and thus confer poor prognosis. The lncRNA, *H19* hypermethylation, and loss of function cause *IGF2* imprinting and hence overexpression in ESCC. The increased *IGF2* levels are associated with tumor grade, lymph node metastasis, and a risk factor for disease progression. *TUG1*, also overexpressed in ESCC, promotes tumor growth, proliferation, and migration and is associated with positive family history and upper segment ESCC and may predict poor clinical outcome. *LOC285194* (LSAMP antisense RNA 3) is however downregulated in ESCC and is associated with tumor size and metastasis and correlates with chemoradiotherapy response. Overexpressed *linc-POU3F3* may be an early detection biomarker of ESCC. In EAC, *AFAP1-AS1* and *HNF1A-AS1* are overexpressed and are associated with proliferation, anchorage-independent growth, and disease progression.

Ten differentially expressed lncRNAs in ESCC tissues were examined for their use as noninvasive diagnostic biomarkers. Of the ten, only *POU3F3, HNF1A-AS1*, and *SYPR4-IT1* were significantly elevated in plasma from ESCC patients. The highest diagnostic performance was provided by *POU3F3* with sensitivity, specificity, and AUROCC of 72.8 %, 89.4 %, and 0.842, respectively. The combination of *POU3F3* levels with serum SCCA improved the performance to AUROCC of 0.926, with sensitivity and specificity being 85.7 % and 81.4 %, respectively. These two biomarkers could detect 80.8 % of patients with early stage disease [46].

5.6.6 Circulating EsCa Protein Biomarkers

Circulating proteins, mostly traditional serum proteins, have been investigated as diagnostic, prognostic, and predictive biomarkers of EsCa.

5.6.6.1 Diagnostic Serum EsCa Biomarkers

Circulating CEA as EsCa Biomarker

Circulating CEA levels are elevated in patients with EsCa, and the diagnostic value have been examined. Similar to other single marker assays, the sensitivity has been low, thus making it unattractive as a screening biomarker. In the meta-analysis by Zhang et al., serum CEA achieved a sensitivity and specificity, and SAUROCC of 8–70 %, 57–100 % and 0.74, respectively [48]. The pooled PLR was 5.95, but it is unattractive as a screening biomarker also partly because of a high NLR of 0.76, suggesting 76 % of test negative patients cannot be excluded as healthy. Similarly, the DOR (a measure of discriminatory test performance) was very low at 9.26.

Circulating Anti-EsCa Antibodies as EsCa Biomarkers

Currently, there are >30 different antibodies against tumor-associated antigens (TAA) that have been investigated for the diagnosis of EsCa. Many of these are single studies, which require further validation work. A few, however, have been investigated by multiple independent workers, though with discrepant results. Many investigations have involved single target assays using ELISA. Of these, anti-p53 antibodies have mostly been targeted. Current technologies, including protein microarrays, immunobead assays, and protein chips, enable analysis of multiple targets in the same assay. Thus, a few assays have employed the use of multiple antibodies as diagnostic panels. Expectedly, sensitivities have been generally low (range 3.9–93.7 %, median ~27 %), but specificities have been acceptably high (range 78.7–100 %, median 98 %) for the single target assays. The use of panel antibodies has improved diagnostic sensitivities (range 23–86 %, median 54.3 %) without compromising specificities (range 89–100 %, median 95.1 %) [47].

Serum p53 antibodies have received meta-analysis for their diagnostic performances. In a meta-analysis of 15 studies comprised of 547 patients and 3406 controls, serum anti-p53 antibodies revealed a significant association with EsCa with a rate ratio of 9.36 and at an overall sensitivity of 91.4 % and specificity of 65.0 % [47]. However, anti-p53 antibodies are not specific to EsCa, because it is of diagnostic value in lymphomas, as well as lung, breast, gastric, colorectal, liver, and ovarian cancers. In another meta-analysis and systematic review of 16 studies (1017 patients and 2877 controls), p53 antibody achieved sensitivity and specificity ranges of 14–60 % and 91–100 %, respectively. The PLR and DOR were 6.71 and 9.60, respectively, but the high NLR of 0.75 precludes its use as a screening test, though the SAUROCC was 0.73 [48]. Apart from anti-p53, other promising EsCa serum antibodies target antigens in NY-ESO-1, MYC, MMP7, HSP70, PRX VI, p16, BMI-1, Survivin, CDC25B, p62, and LY6K.

Circulating CYFRA 21-1 as EsCa Biomarker

A number of studies have examined the diagnostic potential of circulating CYFRA 21-1 levels for EsCa. Because of different study designs, methodological issues, and use of different diagnostic cutoff values, results have been inconsistent. The meta-analysis by Zhang et al. provides insight into the diagnostic performance of CYFRA 21-1 for ESCC [48]. Though data quality was good (85.71 % high quality studies), sensitivity and specificity ranged from 36 to 63 % and 89 to 100 %, respectively. The PLR, NLR, DOR, and SAUROCC were 12.11, 0.59, 22.2, and 0.58. These parameters are unsuitable for its use as a diagnostic biomarker as well.

Circulating SCCA as EsCa Biomarker

SCCA has also been explored as a diagnostic biomarker of ESCC. As a diagnostic biomarker of EsCa, the performance has been dismal. The sensitivity and specificity achieved on meta-analysis of 11 studies ranged from 13 to 64 % and 91 to 100 %, respectively, with a PLR, NLR, DOR, and SAUROCC of 7.66, 0.68, 12.91, and 0.69, respectively [48]. In one study of multiple markers, however, only SCCA improved the diagnostic performance of LY6K, from AUROCC of 0.874 to 0.917 [49].

Circulating VEFGC and Other Proteins as EsCa Biomarker

Studies have explored a diagnostic role for serum VEGFC in EsCa. With only four studies included in a meta-analysis, the pooled sensitivity, specificity, PLR, NLR, DOR, and SAUROCC were 64–85 %, 53–81 %, 2.74, 0.37, 8.12, and 0.81, respectively [48]. These findings suggest a modest diagnostic performance for circulating VEGFC in EsCa.

Other biomarkers examined for diagnosis of EsCa include MMP9 and LY6K. Despite some initial promising results, more studies are required to ascertain their role in EsCa. The cancer-testis antigen, LY6K, is highly expressed in ESCC tissue samples and is associated with poor prognosis. Circulating levels are detected in ~32 % of patients. Elevated circulating antibodies against LY6K can perform at diagnostic sensitivity, specificity, and AUROCC of 81 %, 79 %, and 0.85, respectively.

5.6.6.2 Prognostic and Predictive Serum EsCa Biomarkers

Serum proteins have also demonstrated utility in EsCa prognosis and therapy decision-making. A number of studies have focused on serum CYFRA 21-1 and SCCA levels. Preoperative CYRFA 21-1 and SCCA levels predict survival of patients with ESCC. The median OS was 91.9 months in patients with low, compared to 46.6 months in patients with high preoperative levels. Similarly, low preoperative SCCA levels were associated with 89.7 months vs. 63.7 months for patients with high levels. Multivariate analysis identified these as independent predictors of OS. Importantly, patients with low levels of both biomarkers had perfect (100 %) 5-year survival compared to 27 % for those with double high circulating levels [50]. Elevated CYFRA 21-1 levels were associated with large, deep tumors with lymph node metastasis, and these circulating levels decreased following surgery and have prognostic relevance [51]. CYFRA 21-1 levels correlate significantly with TNM stage. In patients undergoing radiation therapy for stage I–IV EsCa, CYFRA 21-1 values below 3.5 ng/ml at the end of treatment was associated with improved survival and absence of recurrences, while elevated levels were in those with locoregional recurrences [52]. In stage I–IV ESCC patients undergoing chemoradiotherapy, low pretreatment levels of CYFRA 21-1 (<3.4 ng/ml) and CEA (<3.3 ng/ml) were associated with 50 % and 48.3 % complete response compared to patients with high levels, where response rates were 10 % and 42 %, respectively. Low CYFRA 21-1 levels are significant independent predictors of good OS following chemoradiotherapy [50, 53].

Other prognostic circulating biomarkers include MMP9, sE-cadherin, and OPN. For example, high circulating MMP9 levels are associated with advanced tumor stage. The frequency of detection increases with advancing tumor stage, being 36 % in stage I, 75 % in stage II, 90 % in stage III, and in all of stage IV disease patients [54]. The 8 kDa soluble fragment of E-cadherin is elevated in serum samples from ESCC. Multivariate analysis indicates patients with levels below the median experienced better survival than those with elevated levels. Osteopontin is also

overexpressed by EsCa cells and may be associated with disease progression and lymph node metastasis. Together with SCCA, they serve as independent prognostic panel biomarker of survival in patient with ESCC.

5.6.6.3 Serum EsCa Proteomic Biomarkers

A vertical proteomic approach was used to uncover four biomarkers for accurate detection of EAC. By initially profiling tissue samples from BE, BE with dysplasia, and EAC, 11 proteins were tested with ELISA using serum samples. Of the 11, five were significantly increased in EAC compared to controls. However, resistin failed to reach significance in *t*-test used to compare mean EAC vs. GERD values and hence was left out in the final predictive model. A Bayesian rule-learning predictive model with the remaining four biomarkers yielded EAC detection accuracy of 87 % with AUROCC of 0.93 [55].

5.6.7 Circulating EsCa Metabolomic Biomarkers

Using 1H-NMR metabolomics on blood, UHPLC focused metabolomics (on serum amino acids) and multivariate statistical analysis enabled discriminatory metabolites to be uncovered in sera from EsCa patients compared to controls. These metabolites were involved in glycolysis, TCA cycle, amino acid and lipid metabolism, synthesis and degradation of ketone bodies, and energy metabolism [56]. Glycolytic disturbances result in markedly decreased glucose and associated increased lactate levels in blood of cancer patients compared to controls. This finding is partly accountable for by the Warburg effect of aerobic glycolysis. Cancer cells have increased rate of intracellular glucose import, increased rate of glycolysis, reduced pyruvate oxidation, and increased lactate production. Ketones and lipid metabolism are similarly altered. Significantly decreased circulating levels of LDL, VLDL, and unsaturated fatty acids were associated with EsCa, probably due to increased utilization by the cancer cell [56]. This metabolic disturbance was associated with increases in serum ketone bodies, namely, acetoacetate, acetone, and β-hydroxybutyrate. The levels of acetone, the end product of lipid metabolism, were decreased in circulation of cancer patients. Creatine and creatinine levels were elevated in circulation of EsCa patients as well.

The levels of two glucogenic amino acids, glutamate and glutamine, were significantly increased in sera from EsCa patients and may reflect the demands of glucose by the cancer cell. Other amino acids with significant elevated serum levels in patients were histidine, aspartate, cysteine, leucine, phenylalanine, and lysine. Amino acids with decreased serum levels were methionine, tryptophan, and tyrosine. These findings are all indicative of altered amino acid metabolism in EsCa patients. Zhang et al. had previously demonstrated the increased levels of glutamine, phenylalanine, and leucine and decreased methionine, tyrosine, and

tryptophan [57]. Similarly, other consistent findings from these studies are the disturbances in the levels of glucose, lactate, creatinine, and ketone bodies [58].

The work by Zhang et al. in 2012 uncovered differential circulating metabolites between patients with EAC, BE with dysplasia, and healthy controls [57]. The methods used were NMR, followed subsequently by MS, and yet the biomarkers uncovered had some overlap. In comparing EAC patients with healthy controls, 12 MS and 8 NMR metabolites were significantly discriminatory. β-hydroxybutyrate, lysine, glutamine, citrate, creatine, lactate, glucose, and an unidentified molecule were all elevated by NMR in patient samples. Decreased by MS data were linoleic acid, linolenic acid, myristic acid, 5-hydroxytryptophan, tryptophan, tyrosine, leucine/isoleucine, valine, and methionine, while elevated were margaric acid, carnitine, and lactic acid.

Three diagnostic models consisting of only MS data (model 1), only NMR data (model 2), and both MS and NMR data (model 3) were tested for their use in cancer detection. Sensitivity, specificity, and AUROCC for model 1 were 77 %, 86 %, and 0.82, respectively. For model 2, the corresponding findings were 82 %, 88 %, and 0.86, respectively. The performance was enhanced with sensitivity and specificity of 91 % each and AUROCC of 0.95 when model 3 (both MS and NMR data) was applied to the samples.

There were also distinct metabolic profiles between EAC and high-risk patients, defined as patients with BE and high-risk dysplastic lesions. However, of importance was the ability to differentiate between the high-risk patients and healthy controls. Elevated levels of pyroglutamic acid (by LCMS), proline, and lactic acid (by NMR), as well as decreased levels of an unknown metabolite and *N*-acetylated protein, significantly differed between high-risk patients and healthy controls. The only MS metabolite profile performed lower (AUROCC of 0.76) than the NMR metabolite profile (AUROCC 0.80) and failed to enhance performance when combined with NMR data.

The serum levels of several metabolites (~10) including glutamate, histidine, creatinine, lysine, β-hydroxybutyrate, glutamine, tryptophan, tyrosine, methionine, and lactate were consistent with the NMR and UHPLC data from Zhang et al. [56].

5.6.8 Circulating EsCa Cells

Disseminated tumor cells (DTCs) were first used as clinical parameters in the management of EsCa. Iliac crest or sternal sampling and cytokeratin staining were explored at surgery. In one early series, DTCs in iliac crest samples were observed in 37 % of patients with nonmetastatic disease, and these findings were associated with poor survival even after complete tumor removal. While clinical significance could not be determined, two studies suggested that bone marrow samples from a rib, contiguous with tumor, were associated with increased frequency of DTC detection (79 %) compared to iliac crest samples (8 %) [59]. Consistent with parallel-evolution model of metastasis, genome-wide analysis of single

DTCs revealed genetic disparities between DTCs and primary EsCas. Gains at 17q (*HER2* locus) were a marker of DTCs and conferred poor overall survival [60].

Studies of circulating EsCa cells (CEsCaCs) have employed different methods including the CELLSEARCH® method. However, many studies have targeted amplification of genes such as *CEA, KRTs*, and *BIRC5* in circulation. The clinical applications of CEsCaCs are still being uncovered. CEsCaC detection by immunomagnetic separation or CELLSEARCH® technology enabled detection of systemic disease and was useful in treatment response monitoring. Patients who remained positive for CEsCaCs after therapy had poor survival outcomes [61]. CEsCaC enumeration in patients with metastatic EsCa detected the presence of $\geq$2 CTCs per 7.5 ml of blood in 21.7 % of patients, and this was associated with pleural spread and poor prognosis [62]. In patients with metastatic esophagogastric cancer on first-line chemotherapy, baseline CEsCaCs of $\geq$2 were associated with 37.5 % response rate compared to 60 % in those with <2 cells. Additionally, the median progression-free survival was much better in those with <2 CEsCaCs (10.5 months) than in those with $\geq$2 cells (6.1 months) [63].

Molecular targets commonly used for CEsCaCs characterization include *CEA, SCC, KRTs, ΔNp63*, and *BIRC5*. In one case study, CEsCaCs persisted after surgery, and 90 % of them had polysomic chromosome 8 and 20. These findings were associated with metastatic relapse and death, indicating the importance of molecular characterization of CEsCaCs [64].

BIRC5 mRNA was detected in blood from 77 % of patients with EsCa. In multivariate Cox linear regression model, *BIRC5* expression was a significant independent predictor of OS. Elevated postoperative levels were especially associated with shorter OS [65]. In another series, *BIRC5*-expressing circulating cancer cells were detected in 47.2 % of ESCC patients and correlated with depth of tumor invasion, vascular invasion, nodal status, and disease stage. Over a 33-month follow-up period, positive *BIRC5* expression was associated with disease relapse and shorter survival time [66].

CEA expression has been detected in 57.4 % of patients with ESCC, and the frequency of detection was associated with surgery, nodal status, and disease stage and recurrence [67]. CEsCaCs as detected by *CEA* expression increased after surgery. However, 50 % of patients with elevated *CEA* transcript levels (indicating more CEsCaCs) 3 days after surgery had metastatic disease 1 year following surgery, compared with just 14.3 % of those with less CEsCaCs [68]. Initiating tumor cells (ITCs) were detected by *CEA* expression and correlated with *CDH1* expression and disease recurrence. The presence of ITCs significantly correlated with tumor depth, lymph node metastasis, stage, and vascular invasion. Multivariate analysis indicated that ITC and tumor depth were independent predictors of shorter hematogenous disease-free interval. ITCs (as detected by *CEA* expression) correlated with decreases in *CDH1* expression in primary tumors, and this was of prognostic relevance. ITC status and *CDH1* expression were associated with significant longer disease-free interval, hematogenous disease-free interval, and OS compared with those with reduced *CDH1*expression [69].

CEsCaC detection by targeting *CEA* expression in patients with resectable EsCa was significantly associated with advanced stage III/IV disease. Patients with *CEA* mRNA >40 copies per 10^4 normal cells had increased rates of relapse [70]. A large study of the prognostic relevance of CEsCaCs in ESCC patients subjected to curative-intent surgery targeted *CEA* and *SCCA* expression. While detection rate was low in pre- and post-surgical specimens, the presence of CEsCaCs after surgery was one of the independent prognostic factors of disease-free survival, and this was significantly associated with hematogenous and local spread. Decreases in CEsCaCs after surgery, as well as absence of lymphatic invasion, were associated also with response to neoadjuvant chemotherapy [71]. CEsCaC dynamics before and after radiotherapy and clinical significance was determined using *CEA*, *KRTs,* and *BIRC5* expression in blood from patients treated with radical radiotherapy. The posttreatment presence of CEsCaCs was an independent predictor of poor prognosis and inefficient response to radiotherapy [72].

Elevated *KRT8, KRT18*, and *KRT19* (≥75 U/l) levels were detected in sera from 98 % of patients with EsCa, and these elevated levels were associated with disease burden, being significantly increased in metastatic disease compared to localized disease patients. Progressive elevation in *KRT* levels correlated with poor survival in both univariate and multivariate analysis [73]. In patients undergoing curative-intent esophagectomy for ESCC, CEsCaCs as detected by *SCCA* expression were positive in 33 % of patients. Nearly seventy-four percent of patients positive for CEsCaCs developed recurrences. *SCCA* expression on admission correlated with tumor depth and venous invasion [74]. *ΔNp63* expression is specific for squamous cell carcinoma (SCC) and thus was used as a marker of circulating ESCC cells. The detection rate was 52 % in circulation of patients with primary ESCC compared to 60 % of those with postoperative recurrences. The authors suggest that *ΔNp63* is a more sensitive marker than *SCCA* and *CEA* for detection of ESCC cells in circulation [75].

5.6.9 EsCa Extracellular Vesicles

Exosomes and cancer-derived EVs from EsCa cells harbor differential cargo, especially miRNA that uniquely may serve as biomarkers. Additionally, these EVs possess biological activities involved in mediating disease progression. In general, cancer cells may release more EVs than normal cells. Analysis of mucosal extracellular matrix from normal, BE, dysplasia, and EAC samples revealed increased levels of microvesicles (MVs) in BE and EAC than normal mucosa. There were more MVs in EAC than BE (1.9 times) and normal mucosa (5.8 times). Similarly, there were 3.1 times more MVs in BE than normal mucosa [76].

Several miRNAs are carried in exosomes from EsCa patients. MiRNAs in EsCa-derived exosomes may be differentially sorted and packaged. Using Solexa high-throughput sequencing, more known miRNAs were found in EsCa cells (342 miRNAs) than in exosomes (48 miRNAs). Novel miRNAs were of similar

distribution, with 64 in cancer cells and 32 in their exosomes. Of note, the expression profiles of miRNA differed significantly between EsCa cells and their exosomes. This group also found that exosomal miR-21 targets PDCD4 to promote EsCa cell migration and invasion [77, 78].

The differentially circulating levels and hence the diagnostic potential of EsCa-derived exosomal miRNA have been explored. Comparing miRNAs from serum exosomes and matched EsCas with normal tissues enabled several oncomirs (miR-223-5p, miR-223-3p, miR-483-5p, miR-409-3p, miR-196b-5p, miR-192-5p, miR-146a-5p, and miR-126-5p) to be uncovered as exosomal cargo overexpressed in matched tumors. Several other miRNAs (miR-224-5p, miR-452-5p, miR-23b-5p, miR-203-5p, miR-1201-5p, miR-149-5p, miR-671-3p, miR-944-5p, miR-27b-3p, and miR-22-3p) were significantly downregulated and hence rarely detectable in exosomes and matched tumors compared to control tissues [79]. A comparative study design enabled identification of discriminatory miRNAs for EAC. This group profiled 758 miRNAs in circulating exosomes from cancer patients, people with BE, and healthy controls. Differential levels were computed using all possible miRNA permutation ratios. From this, 408 ratios were deferential, of which 179 were significantly discriminatory between EAC and controls (BE and healthy people; AUROCC of >0.70). A multi-marker panel of ratios consisting of RNU6-1/miR-16-5p, miR-25-3p/miR-320a, Let-7e-5p/miR-15b-5p, miR-30a-5p/miR-324-5p, and miR-17-5p/miR194-5p achieved a diagnostic AUROCC of 0.99 for detection of EAC [80].

ESCC-derived circulating miR-21 may be associated with disease progression and potentially of prognostic value. Biologically, exosomes from ESCC patients could induce proliferation of ESCC cells. Quantitatively, the levels of exosomal miR-21 were significantly elevated in patients than control samples, and this correlated with advanced tumor stage, lymph node metastasis, and aggressiveness [81]. This finding is consistent with those of Liao et al. [78]. Of interest, miR-21 was absent in serum cleared of exosomes, indicating almost all of this miRNA is carried in exosomes [81].

EsCa-derived circulating extracellular vesicles have biological activities important for tumor establishment and progression. For example, immune escape is invaluable in cancer progression. EsCa-derived microvesicles could induce naïve B cells to differentiate into TGFβ-producing regulatory B cells that suppressed CD8+ T cell proliferation and activities. These microvesicles were LAMP1+ and contained MMP9 [82].

5.7 Summary

- Carcinogens induce the esophageal epithelium to either undergo dysplastic squamous changes to eventually become SCC or metaplastic dysplastic changes with subsequent development of adenocarcinoma.

- The epithelial metaplasia is caused by gastric acid injury, and hence follows GERD and BE, and is therefore limited to the most distal parts of the esophagus close to the gastric cardia.
- Both EAC and ESCC are characterized by epigenetic and genetic alterations in a multistep fashion.
- The epigenetic and genetic changes, together with the associated altered downstream products (proteins and metabolites), are present in circulation of EsCa patients.
- Exploratory evidence reveals the clinical potential of these circulating biomarkers of EsCa.
- Additionally, circulating EsCa cells are of prognostic relevance in disease management and are currently being evaluated.

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Chapter 6
Gastric Cancer Biomarkers in Circulation

Key Topics

- *Helicobacter pylori* gastritis
- Molecular pathology of gastric cancer (GasCa)
- Screening for *H. pylori* infections
- Circulating GasCa biomarkers
- Circulating GasCa cells

Key Points

- The 5-year survival rate is ~90 % when GasCa is confined to the mucosa but is markedly reduced when the tumor involves the muscularis propria. Biomarkers for early detection will therefore make an impact on patient survival.
- An established etiologic agent of GasCa is *Helicobacter pylori* (*H. pylori*) infection, which accounts for the vast majority of intestinal-type GasCa. The molecular pathology of GasCa involves alterations in *TP53, CTNNB1, HER2, RUNX3,* and importantly *CDH1,* which is silenced via mutations or promoter methylation in almost all diffuse-type GasCas.
- Circulating biomarkers, including targets of *H. pylori*, methylated genes, and circulating GasCa cells, have been in routine clinical practice or being pursued for development of noninvasive products for GasCa management.

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6.1 Introduction

Gastric cancer is forth in incidence and the second leading cause of cancer-related deaths worldwide. The 2012 global incidence and mortality were 631,293 and 468,970, respectively. The 2016 data from the American Cancer Society estimate an incidence of 26,370 and mortality of 10,730 for the US. However, Asia-Pacific, Latin America, and Eastern Europe are some geographic regions with the highest incidence and prevalent rates in the world. For example, the age-standardized incident rates reveal high geographic regions such as Korea, Japan, and China with rates >20/100,000, intermediate regions including Singapore, Taiwan, and Malaysia with rates between 11 and 19/100,000, and low-risk areas such as Northern India, Thailand, Australia, and New Zealand with rates below 10/100,000. There are also pockets of relatively high incidences in the developed world. Los Angeles and Korean men with age-standardized incident rates of >40/100,000 and Alaskan natives with age-standardized incident rates of 36/100,000 are among the highest in the world. These figures imply the need for interventional measures to curtail mortalities from GasCa.

There are various risk factors that predispose an individual to developing GasCa, and these risks relate to the major subtypes of GasCa. Primarily genetic defects underlie the development of diffuse-type GasCa, while environmental and lifestyle factors such as diet and *H. pylori* gastritis are known risk factors of intestinal-type GasCa. Gastric cancer is a major cause of cancer-related deaths globally, partly because of late diagnosis. The 5-year survival is only 30–50 % for advanced stage disease even after curative-intent surgery with lymph node dissection. Local and distant recurrences are common, suggesting ineffective therapeutic targeting of residual disease. Early detection and effective treatment are key to improving the prognosis of GasCa. Because the outlook is good when diagnosed early, there are intensive screening programs in areas with high incidence and prevalence rates. Noninvasive, cost-effective early detection, and other companion diagnostic biomarkers will complement these efforts.

6.2 Screening Recommendations for GasCa

Cancer screening involves employing simple cost-effective tests across an entire healthy population defined to be at elevated risk, such that asymptomatic individuals with the disease or evidence of an impending disease can be identified and treated early. Screening programs are recommended only in situations where the prevalence of the disease is high enough to justify the effort and costs of screening. Thus, in view of the generally low incidence of GasCa in most Western societies, screening is not recommended. However, GasCa screening programs are in place in endemic areas such as Korea, Japan, Taiwan, and China. Currently, many screening programs involve the use of double contrast barium X-ray imaging, photofluorography, and endoscopy. Whereas these procedures are useful in

detecting upper gastrointestinal pathology, they carry a risk of radiation exposure and are uncomfortable and expensive for population screening. Moreover, they detect established disease and thus are not generally useful in early detection strategies.

Similar to many cancers, early detection of GasCa is associated with decreased mortality and better quality of life following treatment. The 5-year survival rate for early localized cancer is over 90 %. Moreover, these early lesions are amenable to less invasive procedures such as endoscopic mucosal resection and/or submucosal dissection. While these procedures have equal benefits as gastrectomy, they offer much better quality of life. Late disease has dismal outcome with less than 50 % 5-year survival rate. Because the etiology and pathogenesis of gastric cancer are well established (especially for the intestinal type), other useful adjunct tests to the above procedures that will enable early detection include noninvasive screening for *H. pylori* infection. Other biomarkers to assess early cancer risk, such as biomarkers of field cancerization (e.g., methylation biomarkers), will be clinically relevant.

6.3 *Helicobacter pylori* and GasCa

H. pylori was first observed in 1979 by an Australian pathologist, Barry J. Marshall, and physician, J. Robin Warren. The organism was subsequently isolated in 1982, after Marshall infected himself, developed gastritis, and isolated the bacilli from his mucosa [1]. Deservingly, Marshall and Warren received the Nobel Prize for Physiology or Medicine in 2005 for their work.

H. pylori infection is strongly associated with intestinal-type non-cardia GasCa. The Centers for Disease Control and Prevention (CDC) estimates that two thirds of the global population is infected with *H. pylori*. The prevalence is estimated to be between 19 and 80 %. However, prevalence rates vary considerably in different geographic regions, reflecting the socioeconomic status of different communities. In general, infection rates are much higher in the developing parts of the world, with prevalence being up to 80 %, than in the developed world where rates are below 40 %. However, only ~1 % of all those infected develop GasCa. Thus, the high prevalent rates in Africa and Northern India are not directly correlated with high GasCa incidences. Other contributing factors facilitate *H. pylori*-mediated GasCa development. Noteworthy is the epidemiologic principle of *agent-host-environment* interactions:

- The virulent factors from the agent, *H. pylori* bacilli, include polymorphisms in *vacuolating cytotoxin A* (*VacA*) and *cytotoxin-associated gene A* (*CagA*).
- Host genetic factors identified include polymorphisms and expression of cytokines (IL-1β, TNFα, IL-10), mucins (MUC-1), and HLA genes.
- Environmental interplay includes increased salt intake, which elevates the risk for GasCa because salt may be a mucosal irritant, while increased intake of fresh fruits and vegetables is protective due to their antioxidant effects.

Another infectious etiology of unknown mechanism in GasCa pathogenesis as yet is Epstein–Barr virus (EBV) infection. The virus is detected in 2–16 % of GasCas, with the majority being tumors from the proximal and middle portions of the stomach. Additionally, expression of EBV-related genes including *EBER1*, *EBER2*, *EBNA1*, *LMP2A*, *BARF0*, and *BARF1* is found in GasCas.

6.4 Molecular Pathology of GasCa

The two major types of GasCa are the intestinal and diffuse types (Table 6.1). While *H. pylori* infection is associated with the intestinal type, diffuse-type GasCa often has a distinct genetic etiology. The mutagenic or epimutagenic effects of *H. pylori* gastritis are noteworthy in the molecular events that mediate gastric carcinogenesis.

6.4.1 Classification of GasCa

Gastric cancer is classified based on tumor anatomic location, gross, and histologic appearance, as well as clinical features. Anatomically, there are distal and proximal cancers, but three types are recognized; type I (distal to esophagus), type II (cardia), and type III (stomach distal to cardia) (Fig. 6.1). Cancers originating from the cardiac region (proximal) and, which may involve the gastroesophageal junction (GEJ) pose a unique challenge as whether to be classified as gastric or esophageal cancers. Indeed, while the incidence of GasCa in general is on decline, GEJ tumors

Table 6.1 Comparison of intestinal and diffuse-type gastric adenocarcinomas

Features	Intestinal (differentiated) type	Diffuse (undifferentiated) type
Frequency	>85 %	~15 %
Age	Older	Younger
Sex distribution	Males > females	Males = females
Regional distribution	High-risk population (e.g., Japan)	Any population
Risk factors	*H. pylori* infection, environment and host factors	Host genetic factors
Region of stomach mostly involved	Non-cardia (antrum, lesser curvature)	Entire gastric epithelium can be affected
Pathology	Ulcerative lesion	Cellular dispersion and thickening of gastric wall (“linitis plastica” or “leather bottle” appearance
Mode of progression	Multistep carcinogenesis	None
Prognosis	Good (early cancers)	Poor

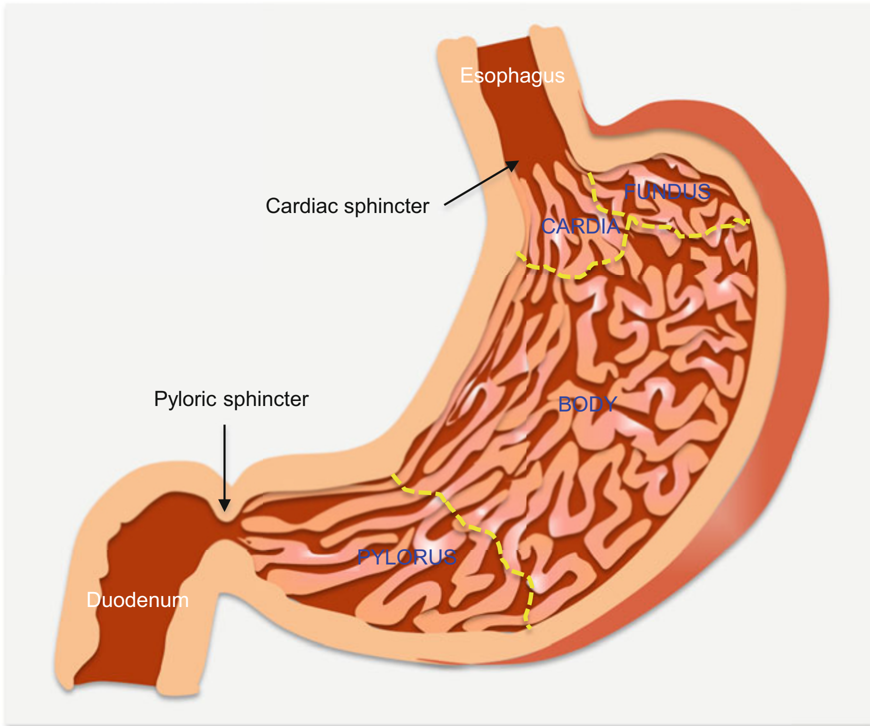


Fig. 6.1 Anatomic parts of the stomach

are on the rise. Histologic classification reveals extensive heterogeneity, while clinical classification as to early or advanced stage disease guides planned treatment protocols.

Clinically, GasCas confined to the mucosa and/or submucosa irrespective of lymph node involvement and tumor size are considered early stage disease. The 5-year survival for these patients is ~90 % compared to <60 % for those in whom the cancer has invaded the muscularis propria. According to Borrmann's classification, advanced stage GasCa exhibit four types of gross appearances or growth patterns. They may be polypoid (type I), fungating (type II), ulcerating (type III), or infiltrating (type IV, also known as linitis plastica) in structure. But these cancers are very heterogeneous at the histologic level.

Histologically, GasCas exhibit architectural and histologic heterogeneity such that even different histologic elements can be found in the same tumor. Lauren in 1965 classified GasCas into three main categories: intestinal, which form the majority (54 % of GasCas), diffuse that is next in frequency (32 % of GasCas), and indeterminate (14 %) types. The intestinal type follows *H. pylori* gastritis and intestinal metaplasia, while the diffuse type has more of a genetic etiology. The WHO, however, identifies four histologic types based on the predominant histologic feature. These are tubular, papillary, mucinous, and signet ring cell

Table 6.2 Classification of GasCa

Lauren (1965)	WHO (2010)
Intestinal type	Tubular adenocarcinoma Papillary adenocarcinoma Mucinous adenocarcinoma
Diffuse type	Signet ring cell carcinoma Other less cohesive types
Indeterminate	~16 different histologic subtypes

(discohesive) carcinomas. The WHO also recognizes uncommon histologic types as mixed carcinomas. In context with Lauren's classification, the papillary, tubular, and mucinous tumors belong to the intestinal type, while the poorly cohesive (signet ring cell) carcinomas belong to Lauren diffuse type. Finally, the numerous rare WHO histologic types (mixed carcinomas) are the indeterminate tumors (Table 6.2).

Tubular adenocarcinomas histologically appear as tubules with intraluminal accumulation of mucin and inflammatory infiltrates. The tubules, may have irregularly distended, fused, or branching architecture. Papillary adenomas are often from the proximal stomach and tend to occur in older people. They also tend to be aggressive with early metastasis to the liver and lymph nodes. Histologically, papillary adenomas form epithelial projections with central fibrovascular tissue. Extensive extracellular mucin (>50 % of tumor) characterizes mucinous adenomas, which are less common than the first two. The cancer cells are either irregularly clustered or form abortive glands. Isolated signet ring cells may be found in these tumors. Signet ring cell carcinomas are usually composed of signet ring cells or other cell types (non-signet ring cell mixture). The non-signet ring cell, but poorly cohesive carcinomas are composed of histiocytes, lymphocytes, and plasma cells.

6.4.2 Genetic Alterations in GasCa

Gastric cancers are mostly sporadic; however, there are evidences of some familial clustering, especially with known cancer-associated syndromes such as familial adenomatous polyposis, Lynch (Peutz–Jeghers), juvenile polyposis, Li–Fraumeni, and hereditary breast–ovarian cancer syndromes, which account for about 1–3 % of all GasCas. As a genetic disease, 10–44 % of GasCas have MSI due to somatic loss of *MLH1*. In addition to established genes such as *CDH1, HER2, TP53, CTNNB1* and *RUNX3*, alterations of several other genes relevant to the pathogenesis of sporadic GasCa include *BAX*, *CDKN2A*, *IGFR2*, *CDX2*, *APC*, and *TGFβR2*. Modifier genes that promote GasCa development in *H. pylori* infections include genetic variations of pro-inflammatory genes such as IL1b, IL-8, IL-10, TLR4, and TNF.

6.4.2.1 *CDH1* Alterations in GasCa

Hereditary GasCa is an autosomal dominant disease with about 30 % of these patients having germline *CDH1* (*E-cadherin*) mutations and deletions. It has a high penetrance such that mutation carriers have over 80 % lifetime risk of developing GasCa. Hence, prophylactic gastrectomy is recommended as a preventive measure for mutation carriers. Because the risk of developing invasive ductal carcinoma of the breast is equally high among women with these mutations, breast cancer screening by mammography is offered beginning at age 35. Mutations in *CDH1* also underlie half of sporadic diffuse-type GasCas, with methylation and reduced expression noted in the remaining 50 %. Thus, almost all sporadic diffuse-type GasCas appear to originate on the background of *CDH1* silencing.

6.4.2.2 *HER2* Alterations in GasCa

Amplification and overexpression of *HER2* is demonstrated in 12–35 % of GasCas. The prevalence is higher (~35 %) in intestinal-type GasCas originating from the proximal stomach and GEJ. All GasCas are tested for *HER2* status, because of the evidence of response to anti-HER2 antibody-targeted therapy and associated good prognosis. The ToGA phase III international trial of combined trastuzumab and chemotherapy revealed a prolonged OS and PFS, suggestive of the effectiveness of trastuzumab in *HER2*-positive GasCas [2].

6.4.2.3 *CTNNB1* Alterations in GasCa

Mutations in *CTNNB1, APC*, and *AXIN1* associated with aberrant WNT/β-catenin pathway activation underlie the pathogenesis of some GasCas. About ~27 % of GasCas harbor *CTNNB1* mutations. Mutations in *CTNNB1*, especially at the APC control regions, make it refractory to regulation by APC, thus leading to β-catenin accumulation, which in complex with TCF in the nucleus induces the transcription of genes such as *CCND1* and *MYC*.

6.4.2.4 *TP53* Alterations in GasCa

TP53 mutations and loss of tumor suppressor functions are common in sporadic intestinal and diffuse-type GasCas. These mutations are early events in cancer development because ~50 % of *H. pylori* gastritis harbors them. Similarly, ~38 % and 58 % of intestinal metaplasia and dysplastic lesions, respectively, have *TP53* mutations. *TP53* LOH occurs in up to 60 %, and mutations in as many as 77 % of GasCas. While the mutational spectrum is diverse, commonly mutated regions are in codons 175, 213, 245, 248, 273, and 285. Of interest, the mutations mostly

involve G:C to A:T transitions at CpG regions. The mutations are more frequent in proximal tumors and in younger patients. They are also more common in advanced than early stage tumors.

6.4.2.5 *RUNX3* Alterations in GasCa

The tumor suppressor gene, *runt-related transcription factor 3* (*RUNX3*), is downstream of the TGFβ signaling pathway. Activated RUNX3 induces the expression of genes such as *CDKN1A* that represses cellular proliferation and proapoptotic *BCL2L11* (BIM). Additionally, RUNX3 inhibits tumor metastasis and angiogenesis by decreasing VEGFA expression. *RUNX3* loss of function via promoter hypermethylation and deletions occur in as many as 45–60 % of GasCas. These alterations are also early in GasCa development because they are present in *H. pylori*-infected gastric mucosa, intestinal metaplasia, and gastric adenomas.

6.5 Screening for *H. Pylori* Infection

H. pylori is a spiral gram-negative microaerophilic flagellate bacillus. Warren and Marshall discovered its carcinogenic effects accidentally in 1982. *H. pylori* is a gastric carcinogen, classified by the WHO as a group I carcinogen. It is the major etiologic agent of GasCa. *H. pylori* infection causes chronic gastritis that can lead to peptic ulceration, atrophic gastritis, intestinal metaplasia, and eventually GasCa. It has been established based on randomized control clinical trial evidences that *H. pylori* eradication significantly reduces the risk of individuals developing GasCa. Therefore in high-risk populations, screening for and treating *H. pylori* infection (though there are issues with this approach such as emergence of resistance strains) is a prudent recommendation. Screening programs currently target adults beginning a decade or two ahead of the period of known risk for individuals developing GasCa within the population. Children are exempt from screening programs because of the high rate of reinfection in endemic areas.

Infection with *H. pylori* usually leads to the production of *H. pylori*-specific immunoglobulins (e.g., IgG and IgA) in a vast majority of patients. Additionally, some infected people have *H. pylori* proteins such as VacA and CagA in circulation. These antibodies and proteins are targets used to develop *H. pylori* serologic tests. There are a couple of issues with the blood serologic assays. First, because of the high background prevalence of infection and hence serologic markers in endemic populations, it is important to develop locally validated serologic assay for different populations. Second, the test is unsuitable as a routine test and is not recommended for evaluating treatment effectiveness, because a positive test result does not differentiate between current and past infection (antibodies may persist for 1–2 years.). The diagnosis of *H. pylori* infection is traditionally by endoscopic visualization and sampling of gastric mucosa for histologic examination,

Table 6.3 Screening for early detection of GasCa

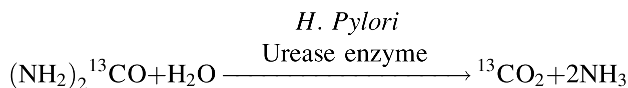
Features	13C-UBT	14C-UBT	Serology	HpSAg	Endoscopy
Sample	Exhaled breath	Exhaled breath	Blood/ serum	Stool	Tissue
Sample acquisition	Noninvasive	Noninvasive	Minimally invasive	Noninvasive	Invasive
Quick to process	No	No	Yes	Yes	No
Cost	Moderate	Moderate	Low cost	Low cost	Expensive
Radiation exposure	No	Yes	No	No	No
Test utility	Detection/ assessment of eradication	Detection/ assessment of eradication	Detection	Detection/ assessment of eradication	Detection/ assessment of eradication
Sensitivity	Up to 98 %	–	Up to 95 %	Up to 95 %	Up to 95 %
Specificity	Up to 98 %	–	Up to 95 %	Up to 100 %	Up to 95 %
Overall performance	Gold standard	Gold Standard	Suboptimal	Suboptimal	Sampling errors due to patchy infections

microbiologic culture, and urease testing (urease positive). However, three noninvasive tests permit cost-effective population-based screening. These are the gold standard urea breath test and the serologic and stool antigen tests (Table 6.3).

6.5.1 Urea Breath Test

This is the gold standard of *H. pylori* testing because it performs much better than all other available tests, including endoscopy, which misses some infections because of the patchy nature of gastric mucosal sampling. The test is used for detection and hence eradication of *H. pylori* infection. Though not globally available, it is the best noninvasive test for *H. pylori* in several endemic communities.

The test, which was initially developed simultaneously by Graham et al. and Marshall and Surveyor, is based on the principle of breath tests used to detect malabsorption [3, 4]. In the case of *H. pylori* testing, it is based on the ability of the organism to produce an active and specific urease in the gut. The test involves an ingestion of a standard 75 mg of carbon isotope-labeled urea after an overnight fast. In the stomach, the urea is broken down by *H. pylori* urease enzyme into carbon dioxide (CO_2) and ammonia. The resulting isotope-labeled $^{13}CO_2$ diffuses into the blood stream and is exhaled through the breath. The level of isotope exhaled, which indicates *H. pylori* activity in the stomach, is detected and measured by mass or infrared spectroscopy.



$$(NH_2)_2{}^{13}CO + H_2O \xrightarrow[\text{Urease enzyme}]{H.\ Pylori} {}^{13}CO_2 + 2NH_3$$

Measurement is done before (baseline) and up to 60 min after urea ingestion. The result is computed as $\Delta^{13}C/^{12}CO_2$ ratio (δ), which is a measure of isotope ^{13}C to non-isotope $^{12}CO_2$. Cutoff levels may be dependent on other factors, such as age, but in general are considered positive when above 2–5 %. ^{13}C is a natural isotope, and therefore samples can be collected anywhere such as in the physician's office and mailed to the laboratory for analysis. However, ^{14}C is a radioactive isotope requiring that testing be done in a controlled nuclear medicine facility. Also because of the long half-life of ^{14}C (5.730 years.), its use is restricted by environmental protection policies.

6.5.2 Stool Antigen Test

The only known niche for *H. pylori* in the gastrointestinal tract is the stomach and duodenum. It is adapted to the acidic environment through the production of urease enzyme that enables the production of ammonia to neutralize the acid. Because of this only known habitat, detection of *H. pylori* antigen in gastrointestinal samples reflects the presence *H. pylori* gastric mucosal infection. The stool antigen test is a direct assay targeting *H. pylori*-specific antigens in stool. Following affinity-purified whole cell lysates from stool, monoclonal, or polyclonal antibodies are used to detect the presence or absence of *H. pylori* in the sample. The procedure can be done with ELISA, which takes about 2 h to complete, or rapid lateral flow chromatography that takes just 10–30 min. The lateral flow method is conducive for small community laboratories that handle just a few samples at a time.

6.5.3 Serum Pepsinogen Test

Serum pepsinogen (PG) levels are influenced by functional and morphological gastric mucosal changes reminiscent of intestinal-type GasCa progression from *H. pylori* infection, gastritis, atrophy, and then to GasCa. Measurement of serum PG as a screening assay for GasCa risk and hence the need for more invasive and expensive procedures have been recommended and been in clinical practice in some endemic areas such as Japan, Korea, and Matsu (an island between Taiwan and mainland China).

There are two biochemically and immunologically distinct PG isoforms produced by gastric mucosal glands: PGI (or PGA) and PGII (PGC). Fundic glands, chief and mucous neck cells produce both types. Additionally, cells of pyloric, cardiac, and duodenal Brunner's glands secrete PGII (Fig. 6.1). Therefore, gastritis,

atrophic, and metaplastic changes in fundic glands lead to decrease serum PGI, while PGII levels remain close to normal because of compensatory production by cells of pyloric and cardiac regions, as well as duodenal Brunner's glands. These changes cause a decrease in the ratio of PGI/PGII. Thus, progressive decreases in this ratio strongly correlate with progressive mucosal changes from normal mucosa to extensive gastritis and gastric atrophy with shrinking numbers of fundic glands.

Complex dynamic changes in PGI/PGII ratio became evident with the discovery of *H. pylori* and its effects on gastric mucosa. *H. pylori* directly and indirectly stimulates PG secretion. Directly, sonicate and lipopolysaccharide from *H. pylori* and indirectly gastric inflammation caused by *H. pylori* stimulate PG release. The effect of *H. pylori* on PG secretion is more pronounced on PGII levels. Thus, infection leads to very high levels of PGII, and hence the ratio of PGI/PGII still remains low, but at a different level from noninfected gastric mucosa. Similarly, with *H. pylori* eradication, PGII levels decrease more than PGI, and thus elevating the PGI/PGII ratio. This has been one source of controversy on the usefulness of the PG test.

H. pylori atrophic gastritis migrates from the antrum proximally toward the cardia, with a progressive reduction in fundic glands. Initially, this progressive mode is associated with more PGII than PGI secretion and hence a reduced PGI/PGII ratio. As disease progresses to atrophy, chief cells are replaced by pyloric glands (metaplasia) leading to much decreases in PGI, but PGII levels remain high; hence, the PGI/PGII ratio is much reduced indicating gastric atrophy and metaplasia. Aside from *H. pylori* status, factors that affect PG release by gastric mucosal cells are age, gender, and histopathologic type of cancer, as well as the distribution and stage of cancer. Different test kits and cutoff values also hinder standardization of the test. However, evidence-based medicine indicates a clear value of PG test as adjunct to imaging-based GasCa screening in endemic areas, especially for the early detection of the intestinal type.

6.5.4 *The Combine Serum* H. pylori *and PG Tests*

This test, also referred to as the ABC (gastritis A, B, C, D) method, is a GasCa risk stratification-screening assay used in Japan. Because no single screening test performs optimally, Dr. Kazumasa Miki [5] developed the combined *H. pylori* IgG antibody and serum PG level assay. For example, the PG test alone is unable to pick up diffuse-type GasCa, which still develops in up to 40 % of patients with negative PG test. The ABC test combines serologic assay for anti-Hp IgG antibody and serum PG levels to stratify individuals into four groups of gastritis or gastric mucosal changes:

- Group A individuals are Hp(−)PG(−), and these individuals have no infection and therefore have normal healthy gastric mucosa. They are not recommended for any further evaluation.

- People in Group B are Hp(+)PG(−). They have mild or no chronic active gastritis (CAG) and only have elevated risk for peptic ulcer. It is recommended that they undergo upper gastrointestinal endoscopy every 3 years.
- Group C individuals are both positive for Hp and PG, i.e., Hp(+)PG(+). They have evidence of CAG and at elevated risk for developing gastric adenoma, hyperplastic polyps, and cancer. Hence they require endoscopy every 2 years.
- Finally Group D individuals are Hp(−)PG(+). They demonstrate the presence of severe CAG, and advanced atrophy, and are therefore at high risk for developing GasCa, especially the intestinal type. Annual endoscopy is recommended for this group.

While, much of the evidence for the diagnostic utility of these biomarkers is from Asian population, some evidence also indicates their possible generalizability to other populations as well. A longitudinal nested case control study of 52 GasCa patients and 104 controls from the Health, Alcohol and Psychosocial Factors In Eastern Europe (HAPIEE) study assessed the predictive value of PGI, PGII, G-17, and IgG antibodies to *H. pylori* (HP-Ab) (named the GastroPanel) for GasCa detection in the Caucasian population. Below baseline cutoff values of all the biomarkers were useful predictors of GasCa, with diagnostic performance ORs of 2.9 for PGI, 9.0 for PGII, 3.3 for PGI to PGII ratio, 1.8 for G-17, and 0.4 for HP-Ab. In multivariate analyses however, PGI/PGII ratio was the strongest independent predictor of GasCa with OR of 2.9 [6].

6.6 Circulating GasCa Biomarkers

Noninvasive biomarkers have been explored in the circulation for GasCa management. These are desirable to complement the current screening efforts for *H. pylori* gastritis in early cancer detection. Thus, being extensively investigated are alterations in the epigenome, noncoding RNA, proteins, and peptides, as well as circulating gastric cancer cells.

6.6.1 Circulating Cell-Free Nucleic Acid Content as GasCa Biomarkers

While primarily focused on the detection of ctDNA through tumor-associated mutations or methylations, a few studies have addressed ccfDNA content variations between GasCa patients and healthy people and their clinical relevance [7–9]. The findings have been consistent with those of other tumors. The levels of ccfDNA are much significantly higher in GasCa patients than controls. As a diagnostic biomarker, the performance of ccfDNA content has been impressive, with AUROCC of 0.75–0.991 [7, 9]. Although elevated ccfDNA is not GasCa specific, these

findings demonstrate the facile detection of GasCa DNA in circulation that can be exploited for various clinical applications.

6.6.2 *Circulating GasCa Epigenetic Biomarkers*

Epigenetic changes in circulation have been explored for their noninvasive diagnostic and prognostic utility in GasCa patients. Indeed, genes commonly methylated in GasCa tissue samples (some of which demonstrate field cancerization) are measurable in circulation of GasCa patients. Their value in early cancer detection deserves further investigation.

6.6.2.1 Circulating GasCa Diagnostic Epigenetic Biomarkers

To test DNA methylation as an early detection biomarker, preoperative serum samples from 106 GasCa patients were subjected to methylation analysis of *CDKN2A* and *CDH1*. Methylation in one or both genes was present in 37 % of the samples including 28 % from early stage cancer patients, suggesting a potential utility in early detection [10]. Subsequent study by this group attempted to increase the sensitivity of the previous assay by incorporating methylation of *RARβ*. The sensitivity was increased modestly from 37 to 48 %. In a follow-up analysis, hypermethylation was demonstrated in three cases with recurrent diseases [11]. Methylation in paired tumor and sera of *DAPK, CDH1, GSTP1, CDKN2B*, and *CDKN2A* were studied in GasCa patients. Hypermethylation of all five genes were frequent in GasCa and matched sera. Over 60 % of serum samples from cancer patients harbored epigenetic alterations, suggesting their utility as screening biomarkers [12]. Methylation of *CDKN2A* promoter in tumor and paired sera from GasCa patients revealed similar methylation frequencies, occurring at a rate of 38 % in primary GasCa tissues and at 26 % in plasma samples. This methylation status, as expected, was very specific to cancer [13]. Preoperative GasCa patient serum samples showed promoter hypermethylation of at least *CDKN2A*, *CDH1*, or *RARβ* in 44 % of cases, including early stage disease patients. Methylation was significantly associated with venous invasion as well [14]. In another series, preoperative serum samples from patients were subjected to methylation analysis of *CDKN2A*, *CDH1*, and *RARβ*, and the results were compared to levels of conventional serum markers (CEA and CA19-9). Hypermethylation was demonstrated in 57 % of patients that showed elevated CEA or CA19-9 levels. No correlation was found between methylation status and elevated serum CEA or CA19-9 levels, indicating complementary use could increase sensitivity for GasCa detection [15]. *ATP4A* and *ATP4B* are downregulated in GasCa, and this is possibly due to intragenic exon 7 (*ATP4A*) and exon 1 (*ATP4B*), but not promoter, hypermethylation. These methylation patterns were detected in ccfDNA in patient plasma samples as well, and are potential GasCa biomarkers [16].

6.6.2.2 Circulating GasCa Prognostic Epigenetic Biomarkers

The prognostic potential of methylated *CDKN2A, CDH1*, and *RARβ* in preoperative serum from GasCa patients receiving curative-intent gastrectomy was evaluated. Hypermethylation of at least one gene was demonstrated in 48 % of patients, but only *CDH1* had prognostic association [17]. Ling et al. evaluated the diagnostic, prognostic, and recurrence prediction value of methylation of *XAF1* in samples from GasCa patients [18]. Methylation and expression of *XAF1* was assayed in 202 GasCa patient tumor tissues, normal adjacent tissues, and paired sera. The downregulation of *XAF1* in GasCa was confirmed, and promoter methylation was demonstrable in as many as 83.2 % of tumor tissues and 27.2 % of normal adjacent tissues (indicative of field cancerization in gastric mucosa), but was completely absent in 88 normal gastric tissue control samples. Similarly, *XAF1* methylation was detected in as many as 69.8 % of patient sera. These methylation patterns performed very well as diagnostic biomarkers, with AUROCC of 0.85 for tissue samples and 0.91 for the serum samples. They also significantly correlated with poor prognosis and predicted recurrences after surgery. *RUNX3* methylation in preoperative and postoperative sera from patients with GasCa revealed a rate of 29 % in preoperative samples, and these levels decreased after surgery. *RUNX3* methylation index (defined as the ratio of amounts of methylated *RUNX3* to *ACTB*) was associated with cancer stage, histology, and lymphatic and vascular invasion [19].

A meta-analysis of DNA methylation in GasCa samples, including whole blood, serum, and plasma, uncovered 77 relevant genes that showed significant differential methylation between cancer and normal samples. The methylation of four genes was detectable in plasma, and seven in serum samples (promoter hypermethylation of *CDKN2B* was present in both media). The general findings were that DNA methylation of 18 genes had prognostic significance, while methylation of *CDKN2A* was significant in predicting outcomes in patients on chemotherapy. Additionally, gene methylation, especially *CDKN2A, DAPK, CDH1*, and *CHFR*, demonstrated field effect in gastric mucosa. The methylation of these genes was significantly different between normal gastric tissues from GasCa patients and those from people without cancer. This meta-analytical study identified the following genes, *DAPK, CDH1, CDKN2A, CDKN2B, SULF1, RPRM, RNF180, SOCS1, SFRP2,* and *MGMT*, to show significant differential methylation in circulation of GasCa patients compared to controls [20]. Multiple studies suggest the diagnostic and prognostic potential of methylated genes in GasCa patients.

6.6.3 Circulating GasCa Genetic Biomarkers

The role of serum MSA in GIST was questioned using LOH at sites of 12 polymorphic markers on five chromosomes. LOH in preoperative serum was detected in

65.4 % of GIST patients but in only one control sample, giving a diagnostic specificity of 87.7 %. This serum LOH strongly correlated with Fletcher risk group. Metastasis was associated with preoperative LOH of two or more markers. The sensitivity and specificity for prediction of relapse were 75 % and 64.1 %, respectively. Serum LOH significantly predicted OS [21]. In a follow-up study by this group, the 12 polymorphic markers were used to determine LOH in ccfDNA, which was detected in 32.6 % of patient samples. An LOH score of two or more markers was observed in 58 % of patients with recurrent disease on CT imaging scans, and this significantly contrasted with only 25 % in those with no clinical evidence of recurrence. However, LOH had no prognostic association after a median 48-month follow-up [22]. The relationship of various clinicopathologic factors to mutated *TP53* in primary tumor tissue and in circulation, as well as circulating p53 antibodies in GasCa patients, was examined. Circulating mutant *TP53* detection was related to progressive tumor depth, and patients positive for mutant *TP53* in both tumor and blood, as well as circulating p53 antibodies, had significantly poorer prognosis [23].

6.6.4 Circulating GasCa Noncoding RNA Biomarkers

Noncoding RNAs, primarily miRNAs and lncRNAs, have been extensively investigated in circulation of GasCa patients. GasCa is characterized by deregulated miRNA expression. Thus, several studies have pursued their differential circulating levels in patients compared to healthy control subjects. Table 6.4 summarizes circulating GasCa miRNAs. MiR-17-92 (miR-17, miR-18, miR-19a, miR-20a, and miR-92) and miR-106-363 clusters are strongly associated with GasCa. Moreover, miR-17, miR-20a, miR-106a, and miR-106b have high sequence homology and therefore may regulate identical genes. These miRNAs have thus shown redundant and overlapping roles in GasCa. A large number of GasCa miRNAs show consistent levels with their tissue expression, and such miRNAs may be passively released or nonselectively packaged in vesicles. However, some miRNAs show discordant levels to their tissue expression. These miRNAs are likely packaged in a selective fashion into extracellular vesicles or actively secreted. Irrespective of the mechanism of release, circulating miRNAs have shown better diagnostic performances than traditional GasCa serum biomarkers.

Table 6.4 Circulating GasCa miRNAs

Increased	Decreased
let-7e, miR-1, miR-17, miR-17-5p, miR-20a, miR-21, miR-27a, miR-27b, miR-34a, miR-106a, miR-106b, miR-175-5p, miR-187, miR-191, miR-196a, miR-199a-3p, miR-200c, miR-221, miR-222, miR-223, miR-335, miR-370, miR-371-5p, miR-376c, miR-378, miR-421, miR-423-5p, miR-451, miR-457, miR-486, miR-744	let-7a, miR-195-5p, miR-218, miR-375

6.6.4.1 Circulating GasCa Diagnostic MiRNA Biomarkers

A number of studies have demonstrated the diagnostic potential of circulating miRNAs in GasCa. They have proven to be early detection biomarkers and shown the ability to differentiate between proximal vs. distal cancers.

Gastric cancer tissue-associated miRNAs (let-7a, miR-17-5p, miR-21, miR-106a, and miR-106b) were assayed in plasma from GasCa patients. While let-7a levels were decreased, the other four miRNAs were elevated in plasma from patients compared to controls. The levels of miR-21 and miR-106b were significantly reduced following surgical resection. While miR-106b was the best diagnostic single biomarker with AUROCC of 0.721, the best overall performance was achieved using the ratio of miR-106a to let-7a (AUROCC of 0.879). The early detection potential of these miRNAs is also suggested by the fact that many patients in this study had stages I/II disease [24]. Zhou and colleagues confirmed the association of miR-106a with GasCa [25]. The levels of plasma miR-106a and miR-17 are significantly much higher in cancer patients, with a diagnostic performance AUROCC of 0.741 for GasCa. However, miR-17 was a much better performer (AUROCC of 0.743) than miR-106a (AUROCC of 0.684). Zhou et al. suggested that both miR-17 and miR-106a were markers of circulating GasCa cells [25]. They showed a significant correlation between circulating miR-17 ($r = 0.912$) and miR-106a ($r = 0.906$) with the number of CTCs. They later identified miR-421 as another marker of circulating GasCa cells, as the levels in blood mononuclear cells were significantly much higher in cancer patients than controls. The diagnostic differentiation of cancer from controls achieved an AUROCC of 0.773 using these miRNAs. MiR-21 is another putative marker of CTCs in GasCa patients [26]. Circulating levels correlate with TNM stage, tumor size, and tissue categories and achieved a diagnostic separation from normal controls with an AUROCC of 0.853.

Global profiling of serum samples from GasCa patients uncovered a number of differentially expressed miRNAs (miR-1, miR-20a, miR-27a, miR-34, and miR-423-5p), with high discriminatory power than single blood markers such as CEA and CA19-9 in cancer detection. As a panel, this miRNA signature had a diagnostic accuracy with AUROCC of 0.879 and 0.831 for the training and validation sample sets, respectively [27]. Out of seven miRNAs differentially expressed between GasCa and controls, three (miR-187*, miR-371-5p, and miR-378) were validated by PCR [28]. In multivariate analysis, however, only miR-378 had an independent diagnostic potential with AUROCC of 0.861 for GasCa [28]. Several circulating miRNAs including miR-21, miR-187, miR-233, miR-371-5p, miR-378, miR-451, and miR-486 are increased in GasCa compared to controls. However, only miR-451 and miR-486 were validated in plasma samples and achieved AUROCC of 0.94 (miR-451) and 0.92 (miR-486) for GasCa detection [29]. Gorur et al. provide evidence for the early detection of GasCa using miRNAs [30]. Of 740 miRNAs profiled in plasma from early stage GasCa patients, only miR-195-5p showed significant downregulation in cancer patients compared to

controls. They suggest this to be a tumor suppressor miRNA important in GasCa initiation and progression.

MiR-223 levels correlate with *H. pylori* infection, and miR-221, miR-376c, and miR-744 are early serum biomarkers that could detect GasCa 5 years before clinical diagnosis. Their diagnostic performance though reached a sensitivity of 82.4 % and specificity of 58.8 % [31]. MiR-199a-3p is significantly elevated in plasma from patients with early stage GasCa compared to controls and those with precancerous lesions, and the levels decreased following surgery. Upon validation using independent samples, miR-199a-3p achieved a diagnostic sensitivity, specificity, and AUROCC of 76 %, 74 %, and 0.818, respectively, for early detection of GasCa [32]. Zhang et al. demonstrated miR-375 to be a putative biomarker of distal GasCa, being significantly decreased in tissues from distal compared to proximal (cardiac) GasCas [33]. Circulating levels are much lower in patients with distal GasCa than healthy controls and those with proximal GasCa. The diagnostic performance for detection of distal GasCas achieved a sensitivity of 80 %, specificity of 85 %, and AUROCC of 0.835.

Meta-analyses have identified circulating miR-21 and miR-223 as potential powerful diagnostic biomarkers of GasCa. In 22 studies on circulating GasCa miRNAs, 35 miRNAs were deregulated in cancer patients, with seven miRNAs reported by at least two studies. However, the most frequently reported was miR-21, which achieved a diagnostic pooled sensitivity of 78 %, specificity of 89 %, and SAUROCC of 0.91 [34]. But it should be noted that circulating miR-21 levels are deregulated in other solid tumors as well. Another meta-analysis of 11 studies yielded a pooled sensitivity, specificity, and AUROCC of 81 %, 84 %, and 0.89 for the diagnostic potential of miR-223 [35]. A validation test performed uncovered that plasma levels of miR-223 were significantly higher in GasCa patients than controls. As a diagnostic biomarker, the sensitivity, specificity, and AUROCC of circulating miR-223 were 70 %, 80 %, and 0.812, respectively. The circulating levels of these miRNAs correlate with expressions in tissues and cell lines.

6.6.4.2 Circulating GasCa Prognostic MiRNA Biomarkers

Circulating miRNAs of prognostic relevance in GasCa include miR-17-5p, miR-20a, miR-21, miR-196a, miR-200c, and miR-335. The postoperative decreases in levels of circulating miRNAs suggest their origin from GasCa. MiRNAs modulated by GasCa surgery include miR-20a, miR-21, miR-106b, miR-196a, miR-199a-3p, miR-451, and miRNA-486 [24, 26, 29, 32, 36]. Their usefulness in determining the completeness of surgical cancer tissue removal, the presence of distant metastasis or micrometastasis, and the need for adjuvant therapy is informative, requiring further validation studies. MiR-21 in preoperative and postoperative plasma from Chinese GasCa patients revealed decreased levels in postoperative samples in all patients. However, the decrease was more dramatic in patients without family history of GasCa (22.1 times lower than preoperative

levels) compared to those with family history (1.76 times lower than preoperative levels) [37]. This decrease was also associated with the degree of differentiation and lymph node metastasis. Kim et al. also found significant increases in serum levels of miR-21, miR-146a, and miR-148a in patients with lymph node metastasis compared to those without [38].

Several miRNAs are informative of survival outcomes in GasCa patients. Komatsu et al. demonstrated high plasma levels of miR-21 in postoperative GasCa patients, and this was significantly associated with poor survival and vascular invasion [39]. In multivariate analysis, increased plasma miR-21 was an independent prognostic factor with HR of 13.4. Circulating levels of miR-200c are also much higher in GasCa patients than controls, and the levels increase with increasing tumor stage from stages I to IV, as well as lymph node involvement. Multivariate analysis identified high levels of miR-200c to be an independent prognostic factor for OS [40]. MiR-196a is elevated in GasCa tissues, and extracellular levels correlate with cellular expression. Circulating miR-196a was associated with relapse. Biologic evidence indicates that miR-196a promotes tumor cell epithelial-to-mesenchymal transition, invasion, and migration [36]. MiR-17-5p and miR-20a are elevated in plasma from GasCa patients and are of diagnostic potential. Additionally, the high circulating levels are associated with TNM stage, differentiation status, and tumor progression. High levels of these miRNAs are significantly correlated with poor OS. MiR-20a is an independent prognostic predictor [41]. Circulating levels of miR-17-5p and miR-21 are elevated and associated with TNM stage and poor OS as well. Additionally, miR-21 is an independent predictor of prognosis. Increased circulating levels of miR-1, miR-20a, miR-27a, miR-34, and miR-423-5p correlate with tumor stage, while plasma miR-199a-3p is associated with lymph node metastasis and TNM stage [27, 42]. But only miR-17, miR-20a, and miR-541 are suggested to be the most relevant GasCa biomarkers with companion diagnostic and prognostic utility.

In conclusion, miRNAs are explored for various utilities in GasCa patients. Specifically, they have been demonstrated to have utility in early cancer detection, differentiation between cardia and non-cardia GasCa, and possible detection of diffuse-type GasCa. The clinical performance of miRNA in GasCa has been variable. However, selecting the best miRNAs as panel biomarker can give optimal diagnostic utility. For example, the combined use of miR-21, miR-218, and miR-223 achieved a sensitivity, specificity, and AUROCC of 84.29 %, 92.86 %, and 0.9531, respectively, in GasCa detection [42]. A panel of five miRNAs (miR-16, miR-25, miR-92a, miR-451, and miR-486-5) appears as an accurate biomarker for the detection of early non-cardia GasCa with sensitivity of 84.1 %, specificity of 90.8 %, and AUROCC of 0.89 [43]. Of equally impressive performance with sensitivity, specificity, and AUROCC of 74 %, 75 %, and 0.818, respectively, in early non-cardia GasCa detection is miR-199a-3p [44]. Moreover, diffuse-type GasCa is associated with circulating miR-103, miR-107, miR-194, and miR-210 in a mouse model [45].

6.6.4.3 Circulating GasCa LncRNA Biomarkers

A few deregulated lncRNA in GasCa tissue samples are reflected in circulation. Upregulated expression and associated increased levels in circulation are *LINC00152* and *H19*, while *PTENP1, LSINCT-5, CUDR*, and *FER1L4* levels are reduced. *LINC00152* levels are significantly higher in gastric juices and plasma samples from patients than healthy controls [46]. As a potential diagnostic biomarker, circulating levels of *LINC00152* achieved a modest sensitivity of 48.1 %, specificity of 85.2 %, and AUROCC of 0.657. The elevated *H19* levels in GasCa patients reduced following surgery, and the performance for GasCa detection had a sensitivity, specificity, and AUROCC of 74 %, 58 %, and 0.64, respectively [47].

FER1L4 may be a tumor suppressor lncRNA (downregulated in GasCa tissues) associated with advanced stage cancer [48]. While preoperative circulating levels in GasCa patients were identical to those in healthy individuals, there was a marked decrease in patient samples 2 weeks after surgery. *PTENP1, CUDR/UCA1*, and *LSINCT-5* levels are significantly decreased in serum samples from GasCa patients compared to healthy people and those with non-GasCa. As a panel, they achieved a sensitivity, specificity, and AUROCC of 74.1 %, 100 %, and 0.92 in GasCa detection. Moreover, the three could detect early GasCa at a sensitivity of 77.8 %, specificity of 97 %, and AUROCC of 0.92, as well as differentiate GasCa from gastric ulcer patients at a sensitivity of 91.7 %, specificity of 83.3 %, and AUROCC of 0.902 [49]. While these pilot discovery findings are impressive, there are however discrepancies in tissue and circulating levels of *CUDR* and *LSINCT-5*, probably due to selective release.

6.6.5 Circulating GasCa Protein Biomarkers

Both traditional serum proteins and antitumor-associated antibodies continue to play a role in GasCa management. Attempts at complementing these are the discovery of differential protein peak signatures and novel proteins for GasCa.

6.6.5.1 Traditional Serum GasCa Protein Biomarkers

Traditional serum biomarkers, including CEA, CA125, CA19-9, CA72-4, and AFP, have been investigated extensively for the clinical management of GasCa. The current synthesis of evidence confirms their relevance in disease management (e.g., prognostication), but not as early detection or diagnostic biomarkers.

The Task Force of the Japanese Gastric Cancer Association reviewed data to ascertain the clinical relevance of CEA, CA19-9, and CA72-4. Of a total of 190 publications inclusive of all three biomarkers, 184 were on only serum CEA and CA19-9. While the positive rates of elevated biomarkers were generally low

(21.1 % for CEA, 27.8 % for CA19-9, and 30 % for CA125), these biomarkers were significantly associated with tumor stage and poor survival outcomes. They were found useful in disease staging, prognostication, and monitoring for local and distant recurrences, especially given the 2–3 months recurrence detection lead time compared to imaging. It was therefore recommended for their use in staging before surgery or chemotherapy.

Elevated AFP levels were useful for detecting liver metastasis, while CA125 and sialyl Tn (STn) detected peritoneal relapse [50]. A large (14,651 patients included) meta-analysis of only CEA found elevated pretreatment CEA levels to be significantly associated with poor prognosis, as determined by OS (HR: 1.716), disease-specific survival (DSS) (HR: 1.940), and DFS (HR: 2.275). Multivariate-adjusted HR analysis found elevated pretreatment CEA levels to be independent prognostic predictor in GasCa patients with OS (HR: 1.681), DSS (HR: 1.900), and DFS (HR: 2.579) [51]. Another meta-analysis has associated CA19-9 levels with advanced clinicopathologic and prognostic features of GasCa. Of 88 studies analyzed, elevated levels of CA19-9 were significantly associated with poor OS (HR: 1.83), DFS (HR: 1.85), and DSS (HR: 1.33). Additionally, high levels differentiated between early and advanced stage GasCa in regard to stage I/II vs. III/IV (OR: 3.36), pT1/T2 vs. T3/T4 (OR: 2.40), lymph node positive vs. lymph node negative (OR: 2.91), metastasis positive vs. metastasis negative (OR: 2.76), and vascular involvement vs. no vascular involvement (OR: 1.66) [52].

Circulating GasCa-Associated Antibodies

Several antibodies against tumor-associated antigens including p62, p16, p53, c-Myc, survivin, Koc, IMP1, IQGAP3, KRT, REG3A, cyclin B1, NY-ESO1, MUC1, DDX53, MAGE, among several others have been associated with GasCa diagnosis, with some demonstrating better performances than the established traditional serum proteins [53–59].

In a systematic review involving 39 published studies, 34 autoantibodies were reported, with the most commonly studied being anti-p53 (13 articles) [60]. Many studies were on single biomarkers with a few evaluating biomarker panels. The diagnostic sensitivities of the single biomarkers as expected were very low, with a median sensitivity of 12.35 % (range, 0–75 %). However, the specificities were acceptably high, with a median specificity of 99.15 % (range, 71.7–100 %). Although anti-MUC1 and anti-MMP7 had the lowest specificities of 83.9 % and 71.7 %, respectively, they achieved the best performances overall, with the best sensitivities of 75 % for MUC1 and 60.5 % for MMP7. Of the 13 studies on anti-p53 antibodies in GasCa, the performances were similar to other anti-TAAs, with sensitivities of 8.1–32.1 % and specificities of 95.25–100 %.

The diagnostic performances of panel biomarkers have been demonstrated with the use of two to 45 autoantibodies. The specificities are generally high (87–100 %), probably because only tumor-bearing patients harbor these antigens. On the contrary, the sensitivities are variable, but in general low (range, 19.3–98.9 %), which may partly be accounted for by many factors including lack of antigen expression by tumors, weak immune system of the host, various effective antitumor immune

mechanisms, and the number of antibodies used in a diagnostic panel. Thus, the sensitivities have increased modestly with those employing ≥7 (range, 52.7–79 %) compared to ≤3 (range, 19.3–22.9 %) biomarkers. Koziol et al. achieved a high sensitivity of 91 % (in cross validation studies) with the use of recursive partitioning on the same dataset from Zhang et al., which achieved an initial sensitivity of 52.7 % and specificity of 89.9 [54, 55]. This data included seven antibodies from six cancer types, breast, colorectal, gastric, lung, prostate, and liver cancers. Recursive partitioning enabled the selection of subsets of the seven that were unique to each cancer cohort. This revealed that no more than three of the seven antibodies were needed for any cancer cohort to achieve desired performances. Zayakin et al. used a T7 phage displayed TAA microarray to uncover 45 relevant autoantibodies for GasCa [53]. This well-designed study included healthy controls matched for age and sex and patients with benign gastric conditions (gastritis and gastric ulcer). The AUROCC for differentiating between GasCa patients and healthy controls, patients with gastric ulcer, and those with gastritis were 0.79, 0.76, and 0.64, respectively.

6.6.5.2 Serum Protein Spectral Peaks as GasCa Diagnostic Biomarkers

A number of studies have explored the use of serum protein peak signatures to differentiate GasCa patients from healthy control subjects. Ebert et al. in 2004 used SELDI-TOF MS, ProteinChip technology, and pattern-matching algorithm to generate 50 decision trees for GasCa classification. The performance achieved a sensitivity of 100 % (89.9 % for early stage disease) and a specificity of 96.7 % [61]. An *m*/*z* peak at 5910 appears to be associated with GasCa. Of three differentially expressed peaks between cancer and controls, a peak with *m*/*z* 5910 was much elevated in serum samples from cancer patients and achieved a diagnostic sensitivity and specificity of 90.9 % and 93.6 %, respectively, and a PPV of 93.8 %. In a follow-up study, differential peak morphology and intensities were noted at *m*/*z* 5084, 5910, 6640, and 8691 in sera from GasCa patients, people with gastritis, and healthy volunteers [62]. The peak at *m*/*z* 5910 was elevated and that at *m*/*z* 8691 was lower in sera from cancer patients compared to healthy individuals. However, in comparison to patients with gastritis, peak *m*/*z* 5910 was still higher in GasCa patients, but peak *m*/*z* 6640 levels were reduced. Lu et al. developed a diagnostic model using five serum protein peaks that achieved a sensitivity of 93.3 % and a specificity of 94.1 % in the training set, and a sensitivity and specificity of 80.0 % and 73.5 %, respectively, in the validation cohort [63]. Poon et al. performed a three-phase study involving a discovery, a diagnostic model development, and an independent model validation [64]. Of 31 SELDI-TOF peaks elevated in sera from GasCa patients, peaks at *m*/*z* 5098, 8610, 11,468, 11,804, and 50,140 were selected for model development because their levels decreased following surgery, suggestive of their GasCa cell origin. The diagnostic model achieved a sensitivity of 83 % and a specificity of 95 % with AUROCC of 0.92 for GasCa detection in the validation studies. Of 17 discriminating peptide peaks, the decision tree with the

best classification included peaks with *m*/*z* 5919, 8583, 10,286, and 13,758. These achieved a sensitivity of 96.7 % and a specificity of 97.5 %. The performance on independent blinded sample sets was equally good with a sensitivity of 93.3 % and a specificity of 90 % [65]. Three protein peaks with *m*/*z* 1468, 3935, and 7560 used as classifier were able to detect GasCa at a sensitivity of 95.6 % and a specificity of 92.0 % in the training set and an equally high sensitivity of 85.3 % and specificity of 88.0 % in the blinded validation data set. Importantly, this study included sera from age- and sex-matched healthy individuals, as well as people with benign gastric lesions and colorectal cancer [66]. Wang et al. demonstrated that six GasCa serum protein peaks at *m*/*z* 3899, 6945, 7035, 8243, 8587, and 9943 could detect GasCa at a sensitivity of 88.5 % and specificity of 97.3 % [67]. Five peptide peaks at *m*/*z* 3300, 4095, 5329, 5910, and 8691 were identified as GasCa biomarkers [68]. Peaks at *m*/*z* 4095, 5910, and 8691 were used in a diagnostic model to detect GasCa at a sensitivity of 92.5 % and specificity of 97.5 %. Xue et al. tested three models for GasCa detection achieving a sensitivity of 96.3 %, specificity of 73.1–84.6 %, PPV of 78.9–86.7 %, and NPV of 94.7–95.7 % [69]. In 2010, Lu et al. identified five serum protein peaks differentially expressed between GasCa and normal controls with sensitivity and specificity of 94.3 % and 93.3 %, respectively, for GasCa detection [70]. In an independent validation serum samples, the five peaks maintained a high sensitivity of 90.3 % and a specificity of 80 %. One peak was more accurate than the rest for early GasCa. Liu et al. found six serum peaks differentially expressed between cancer and controls, with validation performance of 100 % sensitivity and 75 % specificity [71]. From serum proteomics experiments inclusive of gastritis and peptic ulcer patients as controls, five peaks with *m*/*z* 2953, 3267, 5341, 5912, and 5927 were elevated, while four peaks at *m*/*z* 4059, 4213, 4270, and 7160 were decreased in sera from cancer patients [72]. Markedly increased in patient samples was the peak at *m*/*z* 5912, and this had a sensitivity and specificity of 81.25 % and 56.67 % for GasCa detection.

6.6.5.3 Identified Circulating GasCa Proteomic Biomarkers

There are discriminating peaks with identified proteins that have potential in GasCa detection and prognostic prediction.

Circulating GasCa Diagnostic Proteomic Biomarkers

Ebert et al. used magnetic bead-assisted MALDI-TOF MS to identify fibrinopeptide A (FpA) as elevated in sera from GasCa patients. The increased levels were confirmed using ELISA in a large cohort of GasCa patients and people at high-risk for GasCa compared to healthy controls [73]. While demonstrated as a prognostic biomarker, regenerating gene IV (REGIV) is also a potential biomarker of early GasCa. Serum levels are elevated in GasCa patients (median of 8.42 ng/ml in early and 13.12 ng/ml in advanced GasCa) compared to controls (median 4.01 ng/ml). The diagnostic accuracy in a validation set achieved a sensitivity of 94.5 %, specificity of 31.8 %, and an overall accuracy of 60.5 % [74]. From a serum

proteomic profile, out of 13 discriminating peaks, five at *m/z* 2745, 2768, 3402, 6436, and 6629 achieved an AUROCC of >80 % for GasCa detection. The peak at *m/z* 6629 was identified as transthyretin and had an overall sensitivity and specificity of 65.5 % and 92 %, respectively, for GasCa and a sensitivity of 59.4 % for early GasCa detection [75]. Umemura et al. identified a specific peptide peak with *m/z* 2209 that was much more abundant in sera from cancer patients than controls, with a diagnostic AUROCC of 0.715 for stage I GasCa [76]. This peak was revealed to be high molecular weight (HMW) kininogen fragment.

The 14-3-3 protein family members are expressed by all eukaryotic cells, and they participate in diverse cellular functions by interacting with signaling proteins to control cellular apoptosis, cell cycle, cell migration, and spreading, among others. Thus, 14-3-3β is upregulated in GasCa cell line SC-M1, and this has been validated in tissue samples. Elevated serum levels in GasCa patients correlated with number of lymph nodes involved, tumor size, and poor survival [77]. Biologic evidence indicates that 14-3-3β overexpression promotes GasCa cell growth, invasiveness and migration. Indeed, another family member, 14-3-3ζ, is overexpressed in 79 % of GasCa tissues, and levels are associated with the depth of infiltration.

Nine peptides identified through MALDI-TOF MS and bioinformatics analyses could discriminate GasCa from controls at a performance accuracy of 89 % and 88 % in the training and validation sets, respectively [78]. Three of the peptides were fragments of apolipoproteins C-I and C-III. Clinical serum Apo C-I and C-III assay was developed and acquired data correlated with MS data. When used in combination with CA19-9 and CRP, this assay achieved a prediction accuracy of 88.4 % and 74.4 % in the training and validation sample sets, respectively. Ahn et al. selected thirteen proteins from a test set to develop an algorithm that was independently validated on separate sample sets [79]. A number of proteins including EGFR, Pro-Apo A1, TTR, RANTES, VN, D-dimer, IL6, α-2-microglobulin, CRP, and plasminogen activator inhibitor-1 were included in two algorithms that identified GasCa sera at >88 % and >85 % accuracy in the test and validation sample sets, respectively. Liu et al. identified 17 increased and 7 decreased protein spots in sera from GasCa patients compared to controls [80]. The upregulated proteins included plasminogen, apolipoprotein A-IV, kininogen-1, complex-forming glycoprotein HC, complement component C4A, apolipoprotein J, and clusterin. Yang et al. used MALDI-TOF MS to identify 11 differentially expressed proteins, and the two most promising achieved a sensitivity of 95.2 % and specificity of 93.6 % for GasCa [81]. These proteins were identified as fragments of SERPINA1 and ENOSF1.

Loei et al. detected granulin expression in GasCa but not in normal gastric mucosal cells [82]. Serum levels were equally high in patients including those with early stage disease. Serum levels of dickkopf-1 (DKK-1) were significantly higher in patients than controls [83]. With a defined cutoff value of 25 U/ml, a dramatic sensitivity and specificity of 100 % were reported for GasCa detection. A peptide profile differentially expressed between sera from GasCa patients and normal controls led to the identification of four peptides with *m/z* 1467, 1867, 2701, and 2094 that apparently performed at near perfect sensitivity and specificity

for GasCa detection [84]. Peak with *m/z* 1867, 2701, and 2094 were identified as tubulin β chain, thymosin β-4-like protein, and cytochrome b-c1 complex subunit 1, respectively.

Gomes et al. demonstrated proteomic evidence of field cancerization. O-glycosylated protein biomarkers of GasCa have potential for early detection in view of their early association with the disease [85]. Mucin-type carbohydrate antigens (T and STn) in sera from patients with progressive lesions (gastritis and intestinal metaplasia) and controls were examined by immunohistochemistry, and STn was expressed only in intestinal metaplasia. Plasminogen was identified in intestinal metaplasia and GasCa and found to carry STn antigens.

Monoclonal gastric cancer 7-antigen (MG7-Ag) is a putative GasCa-specific biomarker that has been extensively evaluated for GasCa diagnosis. It is being expressed in up to 94 % of GasCa tissues and detectable in up to 60 % of patient serum samples. Ren et al. subsequently demonstrated an 81.4 % sensitivity in GasCa detection [86]. In an endemic Chinese community, serum MG7-Ag had a sensitivity of 77.5 % and a specificity of 95.62 % [87]. MG7-Ag has been found to be a risk factor for predicting progression of precursor lesion to cancer. Combined expression of MG7-Ag and Cox-2 in precursor lesions conferred a risk of 22 times of progression compared to lesions with negative expression. A meta-analytical work covering publications from 1980 to 2013 uncovered 410 published articles of which only seven were of high quality for inclusion [88]. Of the 652 patients, the pooled sensitivity, specificity, PLR, NLR, and SAUROCC were 73 %, 91 %, 8.59, 0.29, and 0.92, respectively, which indicate the diagnostic potential of MG7-Ag for GasCa.

Circulating GasCa Prognostic Proteomic Biomarkers

A classification model using the most discriminating proteins from 32 protein sets could differentiate advanced GasCa patients from controls at a sensitivity of 81 % and a specificity of 90 % [89]. High levels of serum amyloid A (SAA) was significantly associated with poor OS of GasCa patients on chemotherapy. Chan and other workers observed an association between high serum SAA and tumor stage, metastasis, and recurrence, suggestive of its association with dismal outcome [90].

Qui et al. identified five serum protein biomarkers that performed blindly in prognostic prediction (longer $\geq$ 24 months vs. shorter $<$ 24 months survival) at sensitivity of 66.7 % and specificity of 80 %. A specific peak at *m/z* 4474 was significantly elevated in sera from patients with advanced stage GasCa (III–IV) and was associated with shorter survival [91]. Regenerating gene IV serum positivity was a prognostic variable in GasCa, conferring significantly worse outcome than in patients negative for Reg IV [92]. Serum 14-3-3β levels are increased in GasCa patients, and this is associated with tumor size, nodal involvement, and decreased survival rates. 14-3-3ζ is expressed in a majority (~79 %) of GasCas, particularly papillary and tubular adenocarcinoma. Serum levels were associated with histologic type and depth of infiltration [77]. Yasuda et al. observed significant increases in serum levels of soluble vascular adhesion protein-1 in GasCa patients compared

to controls, and the levels decreased with disease progression, and this was associated with poor prognosis [93].

6.6.6 Circulating GasCa Metabolomic Biomarkers

Changes in metabolites are demonstrated in GasCa, mostly using tissue samples. While there are inconsistencies across studies, the Warburg aerobic glycolytic effect is well established. GasCa cells have decreased glucose and pyruvate levels in association with increased lactate and malate. Additionally, the levels of glutamine and valine are higher in cancer than normal gastric mucosal cells. Only a handful of studies have examined circulating metabolite profiles in GasCa patients [94–97]. In general, for carbohydrate metabolites, the levels of 3-hydroxypropionic acid and threonate are elevated, while pyruvate, phosphoric acid, octanoic acid, fumarate, 2-O-mesyl arabinose, and 3-hydroxyisobutyric acid levels are low. Similarly, glutamine, valine, sarcosine, serine, proline, ornithine, pyroglutamate, and asparagine levels are high, while methionine, tyrosine, histidine, tryptophan, leucine, phenylalanine, tyrosine, and hexanedioic acid levels are significantly reduced in GasCa patients compared to controls. For lipid metabolites, circulating levels of cholesterol, cholest-5-en-3-ol, 11-eicosenoic acid, pentafluoropropionate, and cholesta-3,5-diene are high, with nonhexacotanoic acid, trans-13-octadecenoic acid, 9-octadecenoic acid, and 9,12-octadecadienoic acid having low circulating levels.

6.6.7 Circulating GasCa Cells

Circulating GasCa cells (CGasCaCs) have been characterized primarily targeting GasCa-associated transcripts including *CEA, KRTs, MUC1, MUC2, hTERT, MET, EpCAM, MT1-MMP, BIRC5, VEGF, uPAR, TFF1*, and *B7-H3*, as well as ncRNAs such as miR-17, miR-21, miR-106a, miR-200c, and miR-421. The frequency of CGasCaC detection using these molecular methods has been variable (ranges from 9.6 to 71 %). A few studies have employed the CELLSEARCH® method. However, the number of CGasCaCs isolated by this method is much lower than obtained in other cancers (1–2 vs. 6–7 CTCs/7.5 ml of blood for gastric vs. breast cancer). While CGasCaCs may have limited utility in disease detection, they are of prognostic potential.

6.6.7.1 CGasCaCs as Diagnostic Biomarkers

Circulating GasCa cells (CGasCaCs) have been explored, as diagnostic biomarkers, and the results have been inconsistent. In view of this, Tang et al. conducted a meta-

analysis on the diagnostic utility of CGasCaCs [98]. Using their strict inclusion and exclusion criteria, 20 studies involving 1030 patients and 668 controls were analyzed. Many studies included all tumor stages (I–IV). While various methods were used, many studies (85 %) used molecular-based approaches targeting epithelial cell and/or cancer-associated markers. The most frequently used surrogate molecular markers were *CEA* (40 %), *KRT19* (40 %), and *KRT20* (25 %). Less commonly targeted were *EPCAM, hTERT, MUC1*, *cMET, MAGE1, BIRC5, VEGF, MAGE3,* and *GFP*. Only three studies used immunological techniques for CGasCaC characterization. This study established that the pooled sensitivity was low (42 %), but the specificity was as high as 99 % with the SAUROCC being 0.97. From detailed analysis, it was concluded that CGasCaC characterization is unsuitable for GasCa screening. However, given the high specificity, CGasCaC detection can be an ancillary noninvasive adjunct to establishing GasCa diagnosis. Subgroup analysis that looked at the three primary surrogate markers (*CEA*, *KRT19*, and *KRT19*), disease stages and methodology of CGasCaC detection revealed marginal differences in performance. For instance, the sensitivities of the individual markers were equivalent to that of the pooled sensitivity and, as expected, increased marginally in stage IV (63 %) compared to stage I–III (30 %) cases. The three studies that used immunologic methods were more homogenous than the PCR assays, and their pooled sensitivity was as such much higher (82 %) than the PCR assays (35 %).

6.6.7.2 CGasCaCs as Prognosis Biomarkers

Using staining and molecular methods targeting several *KRTs* (2, 7, 8, 18, 19, and 20), *CEA, Ber-EP4, TFF1*, and *MUC2* as a measure of disseminated tumor cells (DTCs) have been reported in patients with resectable GasCa. The presence of DTCs is associated with several clinical parameters including poor metastatic-free survival, OS, and tumor microvessel density (probably mediated by increased VEGFR expression). The ability of DTCs to form tumors has been demonstrated as well. Positive markers of the urokinase plasminogen activator (uPA) system, as well as markers of the extracellular matrix-metalloproteinase inducer (EMMPRIN), can identify DTCs with the ability to form macrometastasis. These markers enable clinically relevant DTCs to be distinguished. But obtaining samples for DTC characterization is invasive. Hence the prognostic role of CGasCaCs has been extensively explored, and findings are fairly consistent. Both CGasCaC enumeration and the use of surrogate markers for detection suggest a role for CGasCaCs in predicting patient outcomes.

CGasCaC detection rate was lower in patients with resectable (10.8 %) than in those with nonresectable (60.2 %) tumors. However, the prognostic role was similar in both cases. Overall survival rate was significantly lower in patients with detectable CGasCaCs. The presence of CGasCaCs was associated with lower relapse-free and OS, and this was an independent prognostic factor in multivariate analysis [99]. Ito et al. used telomerase-specific, replicative-selective, oncolytic adenoviral

agent tagged with GFP gene to detect and enumerate viable CGasCaCs in GasCa patients [100]. Patients with ≥5 CGasCaCs/7.5 ml had significantly worse OS, and the number of CGasCaCs significantly correlated with venous invasion, but not tumor stage.

CGasCaC enumeration in patients with advanced stage GasCa was conducted using the CELLSEARCH® technology and correlated with treatment response. Patients with ≥4 CGasCaCs at 2 and 4 weeks following treatment had significantly shorter median progression-free and overall survival compared to patients with <4 CGasCaCs [101]. CGasCaCs were detected at a much higher frequency (55 %) in patients with metastatic GasCa than in those with nonmetastatic cancer (14 %) using the CELLSEARCH® method [102]. Thus, CGasCaC enumeration by CELLSEARCH® as surrogate biomarkers for the efficacy of chemotherapy in metastatic GasCa patients suggests high CGasCaCs, defined as ≥4 CGasCaCs/7.5 ml detected 2–4 weeks after therapy conferred poor progression-free and overall survival.

As noted, the vast majority of CGasCaC characterization has involved surrogate marker detection by PCR. *Survivin* (*BIRC5*) expression had been measured in a number of studies. *BIRC5*-expressing CGasCaCs were detected at a rate of 45.9 %, and this correlated with Lauren classification, stage, lymph node metastasis, and degree of differentiation. Lymph node metastasis and CGasCaCs were independent prognostic factors of disease-free survival [103]. Bertazza et al. questioned whether CGasCaC detection by *BIRC5*, *KRT19, CEA*, and *VEGF* expression added any prognostic value to the TNM staging system of GasCa [104]. Of the four markers, only *BIRC5* was an independent prognostic factor. Yie et al. [105] had shown that *BIRC5*-expressing CTCs were useful for predicting breast cancer metastasis and recurrence. They extended their study to include gastric and colorectal cancers. CGasCaCs were detected at a rate of 45.4 %. *BIRC5* expression was significantly associated with nodal status, stage, and depth of invasion. A 36-month follow-up indicated that the presence of *BIRC5*-expressing CGasCaCs was a significant independent predictor of cancer relapse and this was much superior to serum CEA.

The detection of *KRT* transcripts has extensively been used as surrogate markers for CGasCaC characterization. *KRT19*-positive CGasCaC detection in patients with advanced stage GasCa was associated with poor prognosis, especially among those unresponsive to systemic chemotherapy [106]. In a multivariate analysis, preoperative detection of at least one of *KRT19*, *CA72-4*, or *CEA* mRNA in GasCa patients was an independent predictor of hematogenous recurrence and lymph node metastasis [107]. Illert et al. examined *KRT20* mRNA as markers of DTCs and CGasCaCs, which was positive in 40 % of the patients [108]. While *KRT20* expression was independent of TNM staging, in multivariate analysis, it stood as an independent prognostic biomarker of GasCa. After sorting out CD45-positive cells, *KRT*-positive initiating tumor cells (ITCs) in the blood and bone marrow were examined before and after preoperative chemotherapy. Forty-four percent of the patients initially positive for ITCs were negative following treatment. Thus, preoperative chemotherapy decreases ITCs in patients with GasCa [109]. In a similar study, CD45-negative and *KRT*-positive CGasCaCs were detected at a rate of

54.4 % before surgery. However, their detection rate fell to 21.1 % after surgery, but this had no prognostic relevance in this cohort of patients with resectable GasCa [109]. The clinical relevance of CGasCaC detected after gastric surgery is unresolved. CGasCaC release following gastric surgery has been associated with both adverse outcomes as well as improved prognosis. Transcript of *KRT19* was suggested to outperform *KRT18, KRT20*, and *CEA* in detection of CGasCaCs. *KRT19* positivity in this cohort was associated with a 5-year survival rate of 50 % compared to 79 % for patients negative for *KRT19*-expressing CGasCaCs. Patients included those who received curative and noncurative-intent GasCa surgery [110]. Wu et al. determined the expressions of *hTERT, KRT19, KRT20*, and *CEA* transcripts as markers of CGasCaCs [111]. While virtually undetectable in blood from controls, each of these markers were demonstrable in >60 % of GasCa patients. However, only *CEA*-expressing CGasCaC detection was significantly associated with clinicopathologic features including tumor size, depth of invasion, vessel invasion, lymph node metastasis, and TNM stage. Additionally, *CEA* expression was a significant independent predictor of postoperative recurrence. These findings led the authors to conclude that *CEA* is a more reliable marker for CGasCaC characterization than the other genes. Postoperative CGasCaC assessment with *CEA* indicated that CGasCaCs are released during gastric surgery but are cleared quickly within 48 h [112].

Other markers of CGasCaCs include membrane type 1 matrix metalloproteinase (*MT1-MMP*) and *B7-H3*. Following microarray identification and validation of *MT1-MMP* as a biomarker of CGasCaCs, bone marrow and blood samples from a large cohort (810 patients) of GasCa patients were examined. The findings from this study were that *MT1-MMP*-expressing CGasCaCs/DTCs were significantly associated with distant metastasis and peritoneal dissemination [113]. Arigami et al. established *B7-H3* as a marker of CGasCaCs by finding significantly increased expression in blood samples from patients compared to healthy controls [114]. High *B7-H3* expression was significantly associated with lower 5-year survival rate compared to patient cohort with low expression. In multivariate analysis, *B7-H3* expression was an independent prognostic predictor in GasCa patients.

6.7 GasCa Extracellular Vesicles

Exosomes may play important roles in GasCa biology, and possible serve as biomarkers, but not many studies have addressed these issues. Baran et al. performed an initial characterization of GasCa-derived EVs [115]. In this pilot study, EVs were significantly elevated in plasma from patients compared to controls. Of interest, these vesicles also contained significantly elevated levels of GasCa-associated molecules including HER2 and MAGE1. Additionally, the levels of CRC6 and CXCR4 were elevated and reduced respectively.

6.8 Summary

- GasCa is a major cause of morbidity and mortality in endemic areas such as Asia-Pacific regions.
- The diagnosis of cancer of the stomach often portends a dismal 5-year survival outcome due to late diagnosis.
- Efforts at primary prevention and early detection of curable disease are therefore being vehemently pursued.
- While diffuse-type GasCa has a genetic etiology (e.g., loss of *CDH1*), intestinal-type GasCa is mostly caused by *H. pylori* gastritis.
- Thus, following the establishment of *H. pylori* as an etiologic agent, validated tests including the urea breath, serologic, and stool antigen tests have been developed for GasCa screening in endemic areas.
- Complementing these tests are other circulating biomarkers that are being harnessed as liquid biopsy of GasCa.
- Promising circulating GasCa biomarkers include gene promoter methylation (e.g., *CDKN2A* and *CDH1*), miRNAs (e.g., miR-17-5p, miR-21, miR-106a, miR-106b, miR-223 and let-7a), other serologic and proteomic biomarkers, as well as CGasCaCs and exosomes.

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Chapter 7
Colorectal Cancer Biomarkers in Circulation

Key Topics

- Colorectal cancer (CRC) screening
- Molecular pathology of CRC
- Circulating CRC biomarkers
- Circulating *SEPT9* methylation as CRC biomarker
- Circulating CRC miRNA biomarkers
- Circulating CRC cells

Key Points

- CRC remains a disease of importance, being among the most commonly diagnosed adult cancers, and a major cause of cancer mortality due to late diagnosis. The dismal 8 % 5-year survival rate can be improved to even 100 % with detection of adenomas likely to progress.
- As one of the major cancers with well-charted molecular pathologic progression model, there are valid biomarkers for use in clinical practice. These include mutations in *KRAS*, *BRAF*, and *APC*, as well as microsatellite alterations.
- Noteworthy, these actionable biomarkers are detectable and measurable in body fluids and are paving the way for noninvasive CRC management. Additionally, novel circulating biomarkers such as miRNAs, protein/peptides, and circulating CRC cells should augment future management of CRC patients.

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7.1 Introduction

Colorectal cancer (CRC) remains a challenge in oncology. First, they remain the most commonly diagnosed cancers, and second, they are the fourth cause of cancer-related mortality worldwide, in spite of the wealth of molecular genetic knowledge on this cancer. The estimated number diagnosed in 2012 was 1.36 million, and as many as 694,000 deaths were recorded for the same year. In the US alone, an estimated 134,490 people will be diagnosed, with 49,190 deaths in 2016. Whereas there are wide geographic variations in CRC incidences, the highest rate occurs in Australia and New Zealand, with the lowest in West Africa. However, the incidence is on the rise globally, and this could partly be accounted for by increased awareness and intensive screening efforts, especially in the more developed world. There are equally geographic variations in mortality rates, with the highest in Central and Eastern Europe and the lowest in West Africa.

The vast majority (up to 85 %) of CRCs are sporadic with no evidence of hereditary components. The risk factors for acquiring somatic gene alterations leading to the development of CRC include age (mean age at diagnosis is 66 years), consumption of diet rich in red meat and unsaturated fat, excessive alcohol use, sedentary lifestyle, and high-energy input. However, the pathophysiology may include a complex interplay between bioenergetics, inflammation, hormonal actions, and even the gut microbiota. These complex and dynamic interacting factors can cause epigenetic and genetic alterations in a vast majority of cells, creating an enlarged area of damaged mucosa consistent with the concept of field cancerization. Subsequently, clonal selection of a CRC cell to expand, grow, and form overt tumors occurs.

Improved surgical techniques coupled with adjuvant chemotherapies and novel biotherapies are making positive impacts on the 5-year survival rates for patients with CRC. However, this benefit is optimal mostly for patients with localized early stage disease (stage I disease patients have a 95 %, while stage II is 82 %). This survival rate drops to 61 % in patients with regional lymph node spread (stage III) and very dismal (8 %) for stage IV-disease patients with distant metastasis. Yet less than 40 % of all patients are diagnosed with early stage disease. The need for improved early detection and effective therapies is urgent and is actively being pursued.

While mostly sporadic, 15–30 % of CRCs have some hereditary components. Achievements made at delineating the molecular genetic alterations in hereditary CRC have helped in the elucidation of the molecular pathology of sporadic CRCs as well. Multiple genes altered in hereditary CRC and also in sporadic cancers include *APC*, *AXIN2*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, *GTBP*, *LKB1*, *STK11*, *MYH*, *PTEN*, *BMPR1a*, and *DPC4*. Despite the plethora of authenticated genetic information on CRC, screening recommendations still rely on fecal occult blood test (FOBT), followed by colonoscopy to visualize and obtain biopsy samples for eventual histopathologic diagnosis. FOBT is a noninvasive assay but suffers from accuracy, while colonoscopy is invasive with possible serious complications. Therefore, validated biomarkers in body fluids should enhance CRC early detection and

management. Thus, the recently FDA-approved methylated *SEPT9* blood test is a step in the right direction that should augment CRC screening efforts.

7.2 Screening Recommendations for CRC

It is a general recommendation that CRC screening begin at age 50 and continue until age 75. However, individuals at higher risk (e.g., people with known hereditary components) than the general population should begin screening at a younger age. Screening of people between ages 76 and 85 should be done based on individual preferences, in consultations with their healthcare provider. There is no recommended screening for people over 85 where the harms outweigh any benefits. Screening should be carried out annually with high sensitive FOBT. Sigmoidoscopy and colonoscopy are offered every 5 and 10 years, respectively. High-risk individuals may need more frequent screening than these recommended timeframes. Newer tests and investigational modalities including fecal immunochemical test (FIT), fecal DNA test, and CT colonography are being considered for possible inclusion in the next recommendation. While laudable, there are compliance issues with CRC screening.

7.3 Molecular Pathology of CRC

Visible outgrowths of the colorectal mucosa (polyps) are very common in middle aged and older people. Although the frequency of polyps in this demographic is as high as >30 %, only <1 % will progress to become cancerous. Pathologically, polyps are classified as nonneoplastic hamartomas (also known as juvenile polyps), hyperplastic polyps, or adenomatous polyps. The majority of CRCs develop from the latter category, which are established precursor lesions. While CRCs may develop in any part of the colorectum, they are more common in the sigmoid colon and rectum (with ~67 % of all cases) than the rest of the large bowel (Fig. 7.1). Grossly, right-sided tumors appear polypoid or fungating exophytic lesions, while the left-sided lesions tend to be annular, constricting the bowel and therefore may present with bowel obstruction or changes in bowel movement such as constipation or diarrhea.

Colorectal cancer is one of the major cancers with well-characterized molecular pathology. The pathogenic mechanisms of hereditary, familial, and sporadic CRCs are initiated and driven by epigenetic and genetic alterations including chromosomal abnormalities involving established oncogenes and tumor suppressor genes. These genetic changes modulate various oncogenic signaling pathways to deregulate normal cellular processes leading to abnormalities in cell proliferation, growth, survival, death, and metabolism. The molecular alterations have given rise to numerous biomarkers for CRC diagnosis, prognosis, and therapy selection.

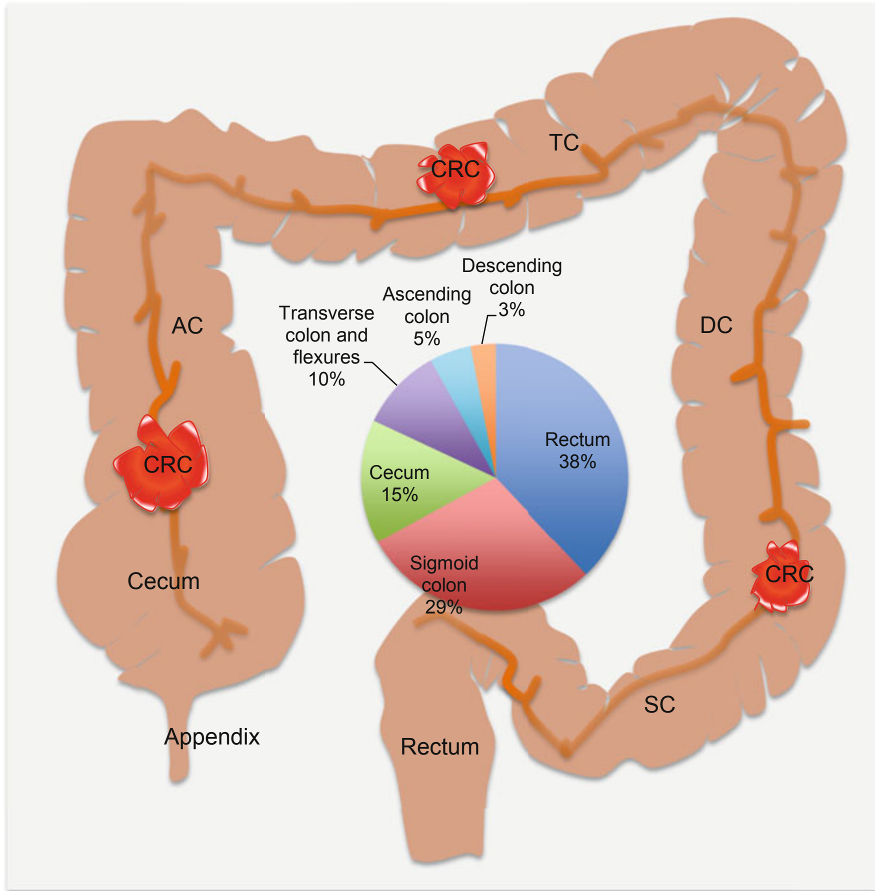

Fig. 7.1 Anatomic locations and frequencies of CRCs. *AC* ascending colon, *TC* transverse colon, *DC* descending colon, *SC* sigmoid colon

Clinically available biomarkers of CRC include predictive *KRAS* codons 12 and 13 and $BRAF^{V600E}$ mutation testing, as well as microsatellite instability (MSI) analysis for disease prognostication. Numerous early stage biomarkers are at various phases of validation.

Colorectal cancers are classified genetically into three subtypes, namely, sporadic, familial, and hereditary CRCs. Sporadic cases occur in individuals with no family history of CRC or any evidence of hereditary (germline) CRC gene mutations. Familial cases occur in people who have at least one first- or second-degree relative, who has been diagnosed with CRC or adenoma but lack any germline mutations or clear evidence of Mendelian segregation. True hereditary CRC, however, by *definition* afflicts individuals who inherit germline mutations in cancer susceptibility genes, such as *APC* and *MLH1*.

Germline and somatic mutations, as well as chromosomal aberrations, underlie colorectal carcinogenesis. The vast majority (up to 90 %) of CRCs involve somatic gene mutations (or familial components without detectable germline mutations), while ~15 % harbor some hereditary component, meaning involvement of first- or second-degree relatives with evidence of germline mutations. Many of those with possible hereditary disease have hereditary nonpolyposis CRC (HNPCC) or Lynch syndrome and familial adenomatous polyposis (FAPs), with the rest being associated with other familial cancer syndromes where CRC is a component of the syndrome. While in the minority, knowledge about hereditary CRCs has provided direction for the study and uncovering of the numerous genetic alterations in sporadic CRCs.

HNPCC occurs in people with mutations in mismatch repair (MMR) genes leading to MSI that affects many tumor susceptibility genes. Cancers are mostly located at the right side (~70 % are proximal to the splenic flexure). The mean age at cancer diagnosis is 45. FAPs evolve in people with germline *APC* mutations. It is characterized by hundreds of adenomatous polyps. Less than 1 % will progress to develop CRC. "Attenuated FAPs" are associated with fewer polyps. Hamartomatous polyposis syndromes such as Peutz–Jeghers, juvenile polyposis, Cronkhite–Canada, and Cowden disease, among others, are associated with elevated risk of CRC.

7.3.1 Molecular Pathology of Multistep Colorectal Carcinogenesis

Colorectal cancer develops in a sequential fashion, with evidence of field cancerization of the colorectal epithelium. Many small polyps (<5 mm in size) are often hyperplastic polyps, which are not major precursors of CRC. However, adenomatous polyps or adenomas can progress to invasive cancers. These adenomas originate from glandular epithelium and are dysplastic lesions with abnormal epithelial differentiation. Their prevalence is about 25 % by the age of 50 but increases to about 59 % by age 70. Adenomas can be prevented from progression by surgical extirpation or polypectomy. Large or advanced adenomas harbor foci of carcinomas, and CRCs tend to have adenomatous lesions within them on histopathologic examination. The genetic condition, FAP, increases the risk of adenomas progressing to CRC, often occurring in early ages (third to fourth decades) if prophylactic colectomy is not performed.

Epigenetic and genetic defects drive adenomas toward overt cancers. The genetic events drive transformation of normal epithelia into benign neoplastic lesions (adenomas), then into invasive cancers, and finally into metastatic cancers with adverse outcomes. The genetic events involved in these steps were initially uncovered and proposed by Vogelstein's group and has been referred to as the classic CRC progression model. This model pertains to progression of tubular and

tubulovillous adenomas. With further elucidation of the molecular progression of CRC, alternative pathways have been uncovered. Serrated polyps (both sessile and traditional serrated polyps) can progress to invasive cancers (these occur primarily in the proximal colon).

The placement of genes in the sequence is not invariant but reflects the frequencies of events at the specific step in tumor evolution. Of note, all the alterations described in the sequence are harbored by only a few CRCs, but at any step in the sequence, the allotted specific genetic event is detectable in many CRCs at a high frequency at that stage, enabling the placement of the gene in that order. The classic tubular adenoma pathway is molecularly characterized by tumors with biallelic loss of *APC*, followed by *KRAS* mutations, loss of *TP53,* and often harbors chromosomal instability (CIN). The serrated adenoma pathway on the other hand displays MSI, CpG island methylator phenotype (CIMP), and $BRAF^{V600E}$ mutations. Tumor progression is further augmented by inactivation of genes with repetitive elements (MSI sites) in their coding sequences (e.g., *BAX*, *ACVR2*, and *TGFβR2*). Figure 7.2a–c summarizes the knowledge of these events in the different pathways. This knowledge is very important as it reflects on biomarker detection in body fluids used for cancer management.

7.3.2 Epigenetic Alterations in CRC

When compared to normal colonocytes, CRC and adenomas exhibit generalized CpG hypomethylation, which is tightly associated with CIN tumors. This general finding contrasts with CpG islands at promoter regions that are often hypermethylated leading to gene silencing. Indeed, many CRC cells exhibit promoter hypermethylation of tumor suppressor genes such as the WNT/β-catenin pathway antagonist, *SFRP,* and hypermethylated in cancer 1 (*HIC-1*). CpG island methylator phenotype (CIMP), while without consensus definition, involves the methylation of multiple gene loci in a particular tumor. The common definition used is promoter hypermethylation of at least three loci from a panel of five markers. Making it even more worrisome is the lack of standardized markers, thus hindering study comparison. A group of sporadic CRCs with CIMP harbors hypermethylation of mismatch repair (MMR) genes (e.g., *MLH1*) constituting the sporadic MSI-H category. This group of tumors harbors *BRAF* mutations and histopathologically are sessile serrated adenomas.

7.3.3 Genetic Alterations in CRC

7.3.3.1 Chromosomal Alterations in CRC

CIN in CRC is characterized by both numerical (aneuploidy) and structural chromosomal changes. Chromosomal changes in CRC include losses at 5q, 8p, 17p, and

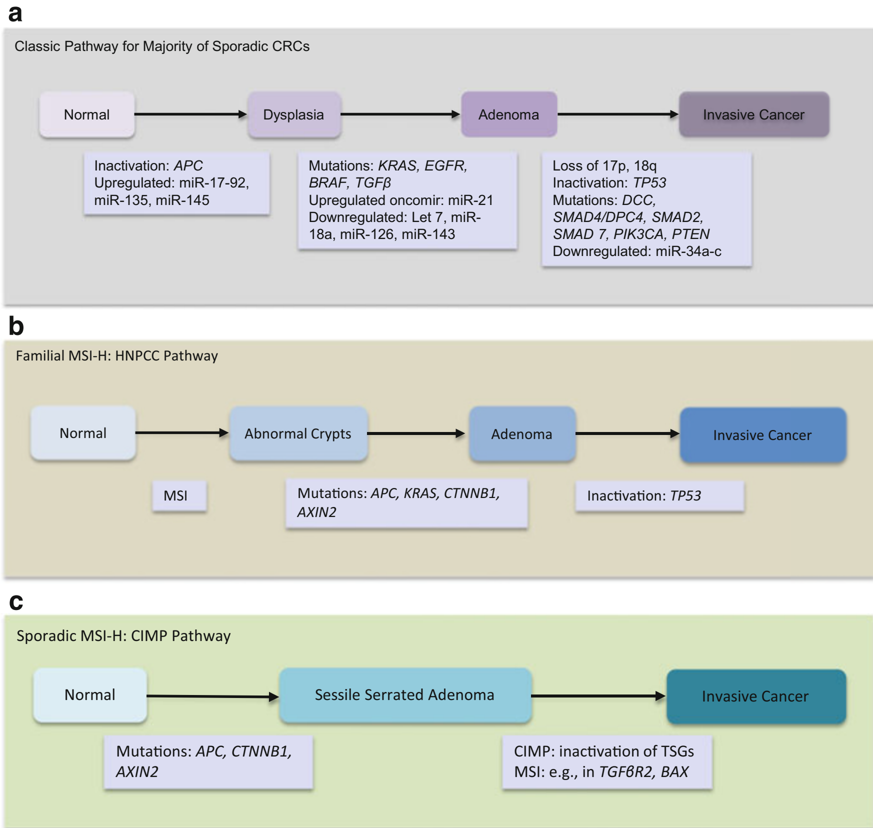

Fig. 7.2 Multistep CRC progression models. (**a**) The classical pathway involving majority of CRCs. (**b**) Familial MSI-H HNPCC pathway. (**c**) Sporadic MSI-H/CIMP pathway

18q and gains at 8q, 13q, and 20q. MSI-H tumors harbor very few or no allelic losses (about 15 % of CRCs without CIN are MSI-H tumors). While the role of CIN in CRC progression is unclear, it may be involved in establishing clonal cell diversity. However, the presence of CIN is a hallmark of poor prognosis. Loss of 18q is demonstrated in ~70 % of CRCs, with frequency distribution associated with tumor size. About half of all large and late-state adenomas harbor deletions, while these are present in only 10 % of small early stage adenomas. This 18q locus harbors important genes implicated in CRC, especially *SMAD4*, as well as *SMAD2* and *SMAD7* involved in the TGFβ pathway. Also at this locus is *deleted in colorectal cancer* (*DCC*), a netrin 1 receptor involved in axon guidance. Often, *DCC* is silenced either by epigenetic mechanisms or loss of 18q21. Credible data suggests *DCC* is a tumor suppressor. Loss of *DCC* is not involved in tumor initiation but has been suggested to promote CRC growth once the tumor is established. The predictive relevance of 18q loss in CRC is under clinical trial NCT00217737 (ECOG 5202 study).

7.3.3.2 Microsatellite Alterations in CRC

Mononucleotide and/or dinucleotide tract MSI accounts for ~15 % of all CRCs. The NCI consensus markers BAT-25, BAT-26, NR-21, NR-24, MONO-27 (these are selected because of their more accurate performances in CRC detection) have been used for CRC MSI detection. MSI was first demonstrated in patients with HNPCC, occurring in all tumors from these patients. Tumors that have gene sequences characterized by >40 % mononucleotide or dinucleotide unstable repeats are classified as MSI-H (or simply MSI+), and tumors without MSI loci are designated as microsatellite stable (MSS). Some tumors, however, harbor 10–29 % unstable MS loci, and these are often designated as MSI-low (MSI-L). While MSI-L tumors have distinct features from MSI-H and MSS tumors, it is not generally accepted whether these are distinct classes of tumors.

In HNPCC, the MMR genes *MSH* and *MLH1* are mutated in ~70 % of tumors. This accounts for the MSI-H phenotype. In sporadic CRCs, loss of *MLH1* expression through promoter hypermethylation accounts for MSI-H tumors. MSI-L tumors have no MMR gene mutations (mechanism unclear). Arguably, MSI-L tumors may not differ from MSS tumors. MSI-H tumors have more favorable prognosis than CIN tumors, and MSI-H and MSS tumors have distinct responses to chemotherapy. Sporadic MSI-H tumors progress via the serrated adenoma pathway and often harbor the $BRAF^{V600E}$ mutation. In contrast, for people with germline MMR gene mutations (as in HNPCC/Lynch syndrome) and MSI-H tumors, disease progression may be determined by *KRAS* but not *BRAF* mutations.

7.3.4 Important Signaling Pathways in CRC

7.3.4.1 WNT/β-Catenin Pathway Alterations in CRC

APC mutations are etiologic factors in familial adenomatous polyposis (FAP) predisposition to cancer. Over 95 % of *APC* mutations in FAP are frameshift or nonsense mutations (more frameshift than nonsense mutations at a ratio of 3:1). These germline alterations are mostly clustered at the 5′ end of the gene, with codons 1061 and 1309 accounting for ~35 % of all mutations. *APC* mutations are early events in 70–80 % of sporadic colorectal adenomas and cancer. Almost all somatic inactivating mutations cause premature truncation of *APC*. These somatic mutations cluster at codons 1309 and 1450 and are found in early adenomas and may be the drivers of adenoma formation. Apparently, both alleles are inactivated in colorectal neoplastic lesions in both FAP and sporadic colorectal adenomas and cancers.

A number of CRCs without *APC* mutations harbor mutations in other members of the WNT/β-catenin pathway. For example, activating mutations in *CTNNB1* (encodes β-catenin) are more associated with colorectal adenomas (12.5 %) than carcinomas (1.4 %). These somatic mutations affecting critical amino acids in the

N-terminal phosphorylation and ubiquitination motifs disrupt APC-mediated degradation of β-catenin. Mutations in *AXIN1* and *AXIN2* characterize many CRCs, although their relevance is yet to be determined. *AXIN1* mutations are predominantly missense substitutions, while *AXIN2* tends to be premature truncating mutations leading to loss of the C-terminal domain. *TCF4* inactivating mutations are found in some CRCs as well.

7.3.4.2 PI3K/AKT Pathway Alterations in CRC

Members of the PI3K/AKT pathway are mutated in >40 % of CRCs. Most frequently affected gene is *PIK3CA*. Specifically, mutations occur in the p110α catalytic subunit. These mutations promote adenoma progression to carcinoma and are found in up to 32 % of CRCs. The negative regulator of the PI3K pathway, *PTEN*, is mutated in 10 % of CRCs, but PTEN protein expression is lost in 15–20 % of CRCs. Mutations are found in ~30 % of MSI-H and in 9 % of CIN CRCs. *PIK3CA* and *PTEN* mutations may have predictive utility in anti-EGFR therapy, because of the cross talk between the EGFR and PI3K pathways.

7.3.4.3 TGFβ Pathway Alterations in CRC

The TGFβ pathway is deregulated in a vast majority of CRCs. Inactivating mutations are observed in receptor genes and intracellular pathway components. Importantly, loss of *SMAD4* occurs in ~50 % of CRCs and is implicated in early stages of CRC development. Thus, it mediates the development of adenomas and drives adenoma progression to carcinoma. Loss of *SMAD4* correlates with lymph node involvement and demonstrates both prognostic and predictive relevance. Other members of the SMAD family members are altered as well. *SMAD2*, also located on 18q, is lost in about ~5 % of CRCs, while *SMAD3*, located on chromosome 15, is inactivated in similar proportions of CRC. Risk alleles (e.g., SNP rs7229639) are putatively found in the *SMAD7* locus (18q21). Inactivating mutations in *TGFβR2* occur in as many as 30 % of CRCs. Functional studies indicate *TGFβR2* mutations drive late adenomas to invasive cancer. While mutations occur in MSI-H cancers, they are observed in only ~15 % of MSS tumors. The coding region of *TGFβR2* has a long mononucleotide tract of adenines that are destabilized by biallelic insertions or deletions in a vast majority (>90 %) of MSH-H tumors. Similarly, *ACVR2A* that encodes a type II pathway receptor is somatically mutated at a polyA repeat in exon 10 in ~85 % of MSI-H tumors and is associated with poor prognosis.

7.3.4.4 *TP53* Pathway Alterations in CRC

TP53 shows LOH in ~70 % of CRCs. Mutations in the other allele, observed in many CRCs, lead to *TP53* inactivation. Many (~85 %) of the mutations are

missense, affecting primarily codons 175, 245, 248, 273, and 282. Nonsense and frameshift mutations are also observed. CRCs without chromosome 17 LOH tend to have mutations affecting both alleles. While mutations are rare in adenomas, they are often observed in later stages in the adenoma–carcinoma sequence. Evidence suggests that *TP53* loss drives adenoma to carcinomas because the occurrence of these mutations followed by LOH of the other wild-type allele coincides with the development of invasive cancers from adenomas.

7.3.4.5 MAPK Pathway Alterations in CRC

Mutations in *KRAS* occur in about 40 % of CRCs. The most frequent hotspots are codons 12 and 13. While these mutations are early events in CRC adenoma–carcinoma sequence, they are preceded by *APC* mutations. The early mutations are maintained throughout tumor progression as they are easily observed in metastatic deposits from the primary tumor. This is important in metastatic CRC patient management where subsequent samples are needed for *KRAS* mutation analysis, because other samples such as body fluids or even archived material will suffice such analysis. *KRAS* mutations also occur at the same frequency (40 %) in adenomas, but the frequency depends on the size of the adenomas. About 58 % of adenomas >1 cm in size harbor *KRAS* mutations, which is similar to the frequency of mutations found in focal adenomas associated with invasive cancers. This finding contrasts with the frequency of 9 % observed in small adenomas <1 cm. Because of their increase in frequency in advanced adenomas, *KRAS* mutations appear to drive CRC growth. Another important gene mutated in CRC is *BRAF*. This gene is mutated in 10–15 % of CRCs. While other mutations occur, the majority is the observed codon 600 single base substitution of glutamic acid for valine ($BRAF^{V600E}$). Similar to other cancers (e.g., melanoma and thyroid cancer), *BRAF* mutations are mutually exclusive to *KRAS* mutations, in support of their dominance or sufficiency in activating the EGF pathway. These mutations occur more often in sporadic MSI-H and CIMP tumors and drive the sessile serrated neoplastic pathway of multistep colorectal carcinogenesis (Fig. 7.2). Consistently, ~50 % of MSI cancers have *BRAF* mutations compared to 5 % of MSS tumors. However, their clinical relevance in CRC risk stratification, prognosis, and treatment predictions is under investigation.

7.4 Circulating CRC Biomarkers

Biomarkers in circulation and stool have been extensively investigated as noninvasive approach to CRC management. While several of these biomarkers remain to be validated for their intended uses, a few including Epi proColon have been launched for CRC risk assessment and diagnosis.

7.4.1 Circulating Cell-Free Nucleic Acid Content as CRC Biomarkers

Multiple studies confirm the value of ccfDNA in the clinical management of CRC patients [1–3]. While not in routine clinical practice, the complementary utility with other serum biomarkers in CRC detection is compelling needing coordinated validation studies.

Serum DII of ALU repeats was much significantly higher in stage I/II ($p = 0.002$) and III/IV ($p = 0.006$) CRC patients than healthy controls. Accuracy as a diagnostic revealed an AUROCC of 0.79 [4]. At least this assay shows potential for CRC detection in a noninvasive manner. Mouliere et al. used atomic force microscopy to visualize ccfDNA in plasma from patients and controls and concluded that the majority of ccfDNA (>80 %) in CRC patients are below 145 bp in size [2]. Using allele-specific blocker qPCR, this group developed a method that could target the smaller ccfDNA fragments simultaneously at multiple levels (concentration, DII, point mutations, and proportion of mutant alleles). This assay provides a powerful noninvasive analysis of ctDNA in cancer patients. Thus, DII defined by ALU247/115 ratio has uncovered a diagnostic potential for ccfDNA in CRC patients [3]. The median absolute serum levels of ALU115 and DII were significantly higher in primary CRC patients than those with polyps and healthy controls ($p < 0.0001$). Similarly, patients with metastatic and recurrent diseases harbored significantly much higher levels than those with primary CRC. The preoperative elevated levels decreased postoperatively. These biomarkers were complementary to serum CEA in CRC detection. Yoruker et al. provide corroborative and confirmatory evidence for the high degree of fragmented DNA in circulation of CRC patients [1]. Two DIIs (ACTB384/106 and ALU247/115) were assessed, and data correlated with circulating nucleosomes. While DII differed between cancer patients and controls, shorter DNA fragments correlated more with circulating nucleosomes, suggesting the presence of highly fragmented nucleosomes (mononucleosomes) in CRC patients.

7.4.2 Circulating CRC Epigenetic Biomarkers

Exploration of epigenetic changes in circulation of CRC patients has mostly focused on known cancer susceptibility genes including *CDKN2A*, *APC*, and *KRAS* and MMR genes such as *hMLH1*. While the clinical importance of circulating promoter hypermethylation of *CDKN2A* is consistent, the reported frequencies of detection vary due to different detection techniques and methodological sensitivities. Promoter methylation of *CDKN2A* in serum as diagnostic or predictive biomarker has been investigated in CRC tissues and matched sera. *CDKN2A* was methylated in 38 % of CRC tissue samples. Serum from 70 % of the positive cases also harbored methylation, and this methylation status was associated with late

Dukes' stage. Another series uncovered *CDKN2A* methylation in 47 % of cancer tissue samples and 30 % of matched sera. In this study, however, methylation in circulation was stage independent, suggestive of its possible use in early detection [5]. Another study by this group found serum *CDKN2A* promoter methylation could detect 69 % of patients with recurrent disease [6]. Later, this group used an MSP to generate numerical methylation scores for circulating *CDKN2A* in CRC patients. The score showed significant correlation with tumor stage, increasing from stage I to IV. Additionally, high scores were significantly associated with lymph node involvement, intravascular invasion, and poor survival [7]. They subsequently developed a more sensitive method for methylation detection in serum, referred to as limiting dilution MSP (LD-MSP). This new assay increased methylation detection sensitivity up to tenfold. Thus, the initial sensitivity of 30 % by MSP was enhanced by the LD-MSP method to 68 % coverage [8]. Further clinical utility of *CDKN2A* methylation in serum a day before surgery, and on follow-up after surgery as a prognostic biomarker, was investigated. Methylation was demonstrated in eight serum samples out of 13 positive tumors and decreased 2 weeks after surgery (two patients with potential for recurrence retained serum methylation). Recurrence a month after surgery was associated with dramatic increases in serum *CDKN2A* methylation. [9]. The prognostic value of ccfDNA in CRC was questioned using dual makers, *KRAS* codons 12 and 13 mutations and *CDKN2A* promoter hypermethylation [10]. *KRAS* was mutated in 38 % of tumors and in 45 % of corresponding plasma samples. The methylation of *CDKN2A* was in 53 % of tumors and 68 % of corresponding plasma samples. An alteration of one or both genes was in 39 tumor samples, of which 37 plasma samples were available, and 70 % of these samples were positive for gene alterations. The 2-year survival was significantly reduced in patients with ctDNA compared to those without, and this prognostic relevance included risk of tumor recurrence.

Other relevant genes with promoter methylation in CRC include *MLH1*, *HLTF*, *APC*, *HPP1*, *THBD*, and *DAPK*. The *hMLH1* and *HLTF* genes have been examined in circulation of CRC patients with potential clinical relevance. Pretreatment sera from CRC patients and controls were assayed for methylation in *APC*, *hMLH1*, and *HLTF*. The serum concentrations of methylated *HLTF* and *hMLH1* significantly differed between cancer and controls (but not for *APC*). Methylation in at least one of these genes gave a sensitivity of 57 % and specificity of 90 % in CRC detection. Methylation in two markers occurred in patients with advanced stage disease [11]. Promoter hypermethylation and gene silencing of *hMLH1* are associated with sporadic microsatellite unstable CRCs. Detection of this methylation in serum achieved a sensitivity of 33 % and a specificity of 100 % for diagnosis of CRCs with MSI [12]. In an evaluation set of sera from controls and patients with localized and metastatic cancer, *HPP1/TPEF*, *HLTF*, and *hMLH1* were identified as useful serum prognostic biomarkers. These genes were then tested on sera from 104 CRC patients. Methylation of the three genes significantly correlated with tumor size. But methylation of *HPP1/TPEF* and *HLTF* was significantly associated with metastasis and tumor stage. Examination of pretreatment sera from 77 patients revealed that methylation of *HPP1/TPEF* and *HLTF* conferred poor prognosis.

Multivariate analysis identified methylation of these two genes in serum to be independent predictors of poor outcome with an RR of death of 3.4 [13].

Methylation of other genes including *DAPK*, *THBD*, and *C9orf50* has been assayed in circulation of CRC patients. *DAPK* methylation frequency was determined in CRC and paired serum samples. Methylation was in 55 % of primary tumors, and positive signal was in 21 % of serum samples [14]. Genome-scale CRC methylation biomarker discovery approach was used on pretreatment plasma/serum from 107 CRC patients and 98 controls to verify selected candidate markers. *THBD* and *C9orf50* were eventually selected for analysis, and both outperformed CEA with high sensitivity and specificity for CRC detection. But *THBD* hypermethylation showed much promise in clinical samples warranting further studies [15].

7.4.2.1 Circulating *SEPT9* Methylation as CRC Biomarker

A gene that has been well studied for noninvasive screening of CRC is the methylation of circulating *SEPT9* gene. Septin 9 (*SEPT9*) located on chromosome 17q25 is a member of the evolutionary conserved septin gene family. Hartwell's work on cell division led to the identification of the septins in yeast. These genes are highly conserved in eukaryotes. They are GTP-binding proteins involved in cytokinesis and also form part of the cytoskeleton. Additionally, septins are involved in cytoskeletal, microtubule, and chromosomal dynamics, vesicle trafficking, cell motility, exocytosis, phagocytosis, cell polarity, apoptosis, spermatogenesis, and platelet functions.

Septins are implicated in neurodegenerative diseases and cancer. They are found associated with protein complexes in Parkinson and Alzheimer's diseases. They are also observed as mixed lineage leukemia (MLL) fusion proteins involved in leukemogenesis. Mutations in *SEPT9*, especially Arg88Trp (R88W) and gene duplications, are associated with hereditary neuralgic amyotrophy (muscle wasting disease with episodes of severe pains). Also, altered expression is associated with several cancers, including cancers of the thyroid, lung, breast, esophagus, liver, pancreas, kidneys, and ovaries. But promoter hypermethylation is specifically associated with CRC and is measurable in plasma and serum for diagnostic purposes.

Stringent sieving criteria involving multiple selection steps in tissue and normal body fluids for analysis enabled three methylated genes to be identified as good candidates for CRC detection in plasma samples. Promoter methylations of these genes (*TMEFF2*, *NGFR*, and *SEPT9*) were tested in 133 CRC and 179 healthy controls. The three genes performed independently at a sensitivity and specificity of 65 % and 69 % for *TMEFF2*, 51 % and 84 % for *NGFR*, and 69 % and 86 % for *SEPT9* [16]. Two large-scale case–control studies of methylated *SEPT9* DNA (*mSEPT9*) in plasma performed later yielded a detection rate of 48 % of CRCs with positive detection in 7 % of the control population in the training set. Blinded validation confirmed this result with sensitivity of 58 % for CRC detection and

10 % in control population. The assay performed at increased sensitivity of 72 % and specificity of 90 % when replicate additional measurements were incorporated. Polyps were detected at a rate of 20 % [17]. An improved *mSEPT9* assay was developed by deVos et al., and this performed at a sensitivity of 72 % and specificity of 93 % in the training set with performance being identical (sensitivity of 68 % and specificity of 89 %) in the test or validation cohort [18].

Another group demonstrated the potential use of *mSEPT9* for screening precancerous lesions [19]. Methylation of *SEPT9* alone and in combination with *ALX4* was investigated in plasma samples. Methylated *SEPT9* was present in 73 % and 29 % of plasma samples from patients with CRC and precancerous lesions, respectively, and 9 % of the control group. When *ALX4* methylation was included, the detection rate of CRC (60 %) and in controls (5 %) was similar to only *mSEPT9* but that of precancerous lesions improved to 37 %. A 2011 study using the improved *mSEPT9* assay increased the sensitivity to 90 % at a specificity of 88 % for CRC detection [20]. This assay identified early stage disease at a rate of 87 %. Cancers of all stages and locations were detected. Twelve percent of adenomas were also detected in a prospective study. A Hungarian study confirmed Warren et al.'s work that detection is unlimited to anatomic site of the cancer. They compared *mSEPT9* to conventional gFOBT and serum CEA assays. Methylated *SEPT9* outperformed both conventional tests. The *mSEPT9* assay achieved a sensitivity of 95.6 % in detecting stage II–IV cancers and 84 % of stage I. Both left-sided (96.4 %) and right-sided (94.4 %) cancers were detected at the same rate [21].

The PRESPT clinical study evaluated the utility of *mSEPT9* in plasma for CRC screening in asymptomatic targeted (50+) population [22]. This multicenter (32 centers) US and German study included 7941 participants of which 53 developed CRC. Samples were blindly assayed in three laboratories with primary focus on sensitivity and specificity for CRC and adenoma detection. This study yielded a sensitivity of 48.2 % for stages I–IV cancers and 11.2 % for advanced adenomas. Specificity was however as high as 91.5 %. Because of the low sensitivity, especially for early stage disease and precancerous lesions, this assay requires some improvement prior to clinical use as a screening tool for targeted population. However, because of the multiple successful case–control and clinical trial studies, this assay (known as Epi proColon) has been approved by the FDA for CRC screening in the average-risk (50–85 year) population.

7.4.3 Circulating CRC Genetic Biomarkers

There are established genetic alterations in CRC initiation and progression. Some of these genes, notably *KRAS*, *APC*, *CDKN2A*, and *TP53*, have been explored in circulation of cancer patients for possible noninvasive translation.

Kopreski et al. first demonstrated the clinical potential of circulating *KRAS* mutations in CRC patients [23]. They detected mutant *KRAS* in plasma or serum from patients with advanced stage disease. To ascertain its potential clinical utility, a

high concordance (83 %) between *KRAS* codon 12 mutations in ccfDNA and tissue samples from CRC patients was later reported in a large study [24]. *KRAS* mutations were detected in plasma of some individuals without colonoscopic evidence of cancer. However, these individuals had high-risk indices for developing CRC, suggesting its relevance as an early risk prediction biomarker. Mutations detected in plasma, coupled with CA19-9 assay, enabled a detection sensitivity of 90 % for CRC. For adenomas, *KRAS* mutations had a low detection sensitivity of 35 % [24].

The prognostic role of *KRAS* (codons 12 and 13) mutations and *CDKN2A* promoter hypermethylation in plasma was examined by Lecomte et al. using mutant allele-specific amplification and MSP [10]. *KRAS* mutations were detected at a rate of 45 %, while *CDKN2A* hypermethylation was observed in 68 % of plasma samples. Of the 39 tissue samples assayed in this study that showed either one or both alterations, 37 plasma samples (~95 %) were informative as well. Seventy percent of these samples showed tumor-specific alterations in plasma. Circulating tumor DNA correlated with poor OS and recurrence-free survival.

Circulating *KRAS* mutations as prediction of disease relapse have been explored with important clinical implications. The relevance of persistent *KRAS* mutation in circulation in postoperative patients was noted by Lindforss et al. [25]. Plasma *KRAS* mutation could be detected in pre- and early post-operative samples in all stages of CRC. While persistent circulating mutant KRAS was not associated with recurrence in this series, the authors still suggested its possible use for postoperative follow-up and early detection of recurrences. Thus, Frattini et al. targeted recurrences on follow-up, using ccfDNA, which decreased in tumor-free patients after surgery but remained elevated in those with recurrences or metastatic disease [26]. This data was supported by the detection of *KRAS* mutations and *CDKN2A* promoter hypermethylation in these samples as well. This group later examined *KRAS* mutations and *CDKN2A* promoter hypermethylation in primary CRC and matched multiple plasma samples at time of surgery and follow-up [27]. Expectedly, total plasma DNA levels were higher in CRC patient samples than in controls. In tumors with *KRAS* mutations and *CDKN2A* promoter hypermethylation, similar findings were observed in their plasma. Plasma DNA levels decreased after surgery but were high in patients with recurrences or relapse. Additionally, *KRAS* and *CDKN2A* alterations were associated with relapse following surgery. This study suggests combined qualitative and quantitative analysis for CRC diagnosis, monitoring for disease-free status, and possible relapse after surgery has potential. There is known acquired resistance to anti-EGFR antibodies during treatment of patients with wild-type *KRAS* genotype. *KRAS* mutation screening of sera from CRC patients on panitumumab (anti-EGFR antibody) monotherapy revealed that 38 % of patients with initial tumor wild-type *KRAS* developed *KRAS* mutations that were detectable in serum samples 5–6 months after treatment. But, mathematical modeling indicated these subclones harbored those mutations prior to the start of therapy [28]. This finding suggests the need for analytically sensitive technologies to uncover such clones prior to therapy.

Gocke et al. first detected mutations in *APC* and *TP53* in plasma from patients with CRC and adenomas [29]. Hsieh et al. focused their examination on mutations

in genes involved with the progressive model of CRC, i.e., *APC*, *KRAS*, and *TP53* in 118 CRC patients and related these to prognostic parameters [30]. Mutations in *APC* and *TP53* correlated with lymph node metastasis and TNM stage. Positive presence of at least one mutation was associated with depth of tumor invasion, lymph node metastasis, and TNM stage. These biomarkers were also related to postoperative metastasis. Mutations in *TP53* were detected at a frequency of 14 % in patient sera using fluorescence-based PCR and SSCP assays. However, these circulating *TP53* mutations were associated with advanced stage tumors with liver metastasis [31]. In another series, patients with advanced stage CRC had detectable mutant *APC* molecules in plasma (median *APC* molecules in plasma were 47,800/ml of plasma with 8 % being mutant molecules). In over 60 % of early disease states, mutant *APC* was found at lower levels of 0.01–1.7 % of total *APC* molecules, suggesting increasing release with progressive disease. Importantly, this study provides evidence of using sensitive technologies for early detection and disease tracking [32]. Diehl et al. compared the detection of mutant DNA molecules in stool and plasma using the same technology (BEAMing) [33]. The detection rate was 92 % in stool compared to 50 % of plasma samples, suggesting stool is superior to plasma for CRC mutation detection. Tumor mutations were mostly in small (150 bp) DNA fragments. The ability to reliably detect tumor mutation in circulating cell-free DNA suggests such DNA measurements are useful for monitoring tumor dynamics following surgery or chemotherapy [33].

7.4.4 Circulating CRC Coding RNA Biomarkers

The mRNA of *CEA* and CEA-related cell adhesion molecule (*CEACAM*) are some circulating transcripts studied for CRC detection. As diagnostic biomarkers, the sensitivities have been modest, ranging from 32 to 69 %. Specificities have equally been problematic, being dependent on controls used in various studies. For instance, specificities have been below 44 % when control samples were from patients with irritable bowel diseases and up to 100 % when considering only healthy individuals without colorectal pathologies. Panel studies involving *CEA* and *KRT* mRNA have improved CRC detection, with sensitivities of between 56–83 % and specificities of 76–100 %. The transcripts of the two epithelial cell genes (*KRT19* and *CEA*) were assayed in plasma from CRC patients and correlated with CTCs and tumor features. In comparison to controls (at 4 % detection rate), *CEA* mRNA was detected in 32 % of CRC patient samples. Similarly, *KRT19* mRNA was detectable in 20 % and 73.6 % of samples from controls and cancer patients, respectively, and these differences were significant. Both biomarkers were associated with advanced stage disease and CTCs [34].

Telomerase activity is increased in ~90 % of CRCs; hence, the mRNA of one component of telomerase, *hTERT*, has been assayed in plasma samples from CRCs. In one series, normalized median levels of *hTERT* transcripts in preoperative samples were significantly higher in CRC patients (11.62) than controls (0.29).

Importantly, 82 % of CRC patients had *hTERT* mRNA levels higher than the maximum value for controls. As a diagnostic, plasma *hTERT* mRNA achieved a sensitivity of 98 % but at a lower specificity of 64 % [35]. Tumor, adjacent noncancerous mucosa, as wells as preoperative plasma from patients with various stages of CRC were assayed for *hTERT* expression [36]. All *hTERT* mRNA (complete gene) and mRNA encoding functional protein were targeted. Both transcripts, as expected, were strongly correlated and were significantly much higher in tumor than noncancerous tissues. Both also increased in relation to tumor progression. All plasma samples, except two, were positive for *hTERT* transcripts. Using a specified cutoff value, the complete *hTERT* transcript achieved a sensitivity of 92 % at a specificity of 100 % for CRC detection. Also, plasma levels correlated with tissue levels of expression [36]. Both cell-free RNA (cfRNA) content and *hTERT* mRNA expression were measured in plasma from rectal cancer patients as a predictive biomarker of response to preoperative chemoradiotherapy. Baseline and post-chemoradiotherapy plasma samples were examined. In univariate analysis, gender, baseline levels of cfRNA, post-therapy levels of *hTERT* and cfRNA, and the difference between baseline and post-therapy levels of both biomarkers were significant response predictors. In multivariate analysis, however, post-therapy cfRNA and the difference between post-therapy and baseline levels of cfRNA independently predicted response [37].

β-catenin is involved in CRC pathology. The mRNA of *CTNNB1* was measured in plasma from patients with CRC, colorectal adenomas, and healthy controls. Median *CTNNB1* mRNA levels were much higher in cancer patients than people with adenomas, and also much higher in adenoma patients than in healthy controls [38]. Transcript levels correlated with tumor stage. In 84 % of CRC patients, the mRNA levels of *CTNNB1* decreased significantly after surgical tumor removal [38].

Metastasis-associated in colon cancer 1 (*MACC1*) is a gene reported to have a strong prognostic relevance for CRC metastasis, enabling early identification of high-risk patients for alternative treatment [39]. The mRNA of *MACC1* was measured in blood samples from 300 CRC patients. Levels were significantly higher in all stages of CRC patients compared to controls. The elevated levels were associated with metastatic CRC and significantly correlated with poor survival. For newly diagnosed patients, *MACC1* and *S100A4* (WNT/β-catenin target) transcripts enhanced prognostic prediction. Other potentially informative transcripts for CRC detection include *guanylyl cyclase* that achieved a sensitivity of >80 % at a specificity of 95 % for early stage disease [40] and *tumor-associated antigen L6* mRNA that performed at over 80 % sensitivity at a specificity of 100 % [41].

7.4.5 Circulating CRC Noncoding RNA Biomarkers

Several miRNAs demonstrate deregulated expression in CRC and may contribute to the adenoma–carcinoma sequence of disease progression. MiR-21, miR-29, miR-34, miR-92, miR124a, miR-130b, miR-139-3p, miR-155, miR-224, and miR-378 have all shown differential expressions and target important genes involved in oncogenic signaling.

7.4.5.1 Circulating CRC Diagnostic miRNA Biomarkers

MiRNAs have been explored as possible CRC risk prediction biomarkers. Inflammatory bowel disease (IBD) including Crohn's disease is an established risk factor for development of CRC. MiRNAs are altered in these diseases and may serve as early detection biomarkers and possible risk predictors. Eight miRNAs were found differentially expressed in sera from pediatric patients with Crohn's disease compared to controls, including patients with celiac disease. In particular, miR-16 and miR-484 were more informative, with miR-16 alone achieving a diagnostic sensitivity and specificity of 74 % and 100 %, respectively, while miR-484 sensitivity and specificity were at 83 % each. Individually, these miRNAs could correctly classify 84 % of Crohn's disease patients compared to conventional markers such as anti-*Saccharomyces cerevisiae* antibodies (ASCA) (70 %) and CRP (76 %) [42].

MiR-29a is associated with tissues of the small and large intestines. Not surprisingly, therefore, plasma miR-29a and miR-92a have been investigated as CRC biomarkers, and can differentiate CRC patients from controls. Ng et al. performed an initial study that involved biomarker discovery, authentication in plasma and tissue samples, followed by independent validation in a large number of patients and controls [43]. Five of 95 miRNAs were upregulated in plasma and tissue samples. MiR-17-3p and miR-92a were significantly elevated in CRC patient samples, and levels reduced following tumor removal in ten patients. Importantly, levels of plasma miR-92a could differentiate CRC patients from controls including healthy individuals and people with gastric cancer and IBD with AUROCC of 0.885 (sensitivity of 89 % at a specificity of 70 %). Further analysis revealed their potential use as early diagnostic biomarkers, because they were elevated in stage I disease, and this achieved a diagnostic performance of 89 % sensitivity and 90 % specificity. Individually, these two miRNAs had AUROCC of 0.717 for miR-17-3p and 0.885 for miR-92a [43]. In another series, of 12 miRNAs examined and found increased in CRC, miR-29a and miR-92a were identified as biomarkers of advanced CRC and advanced adenomas [44]. MiR-29a had an AUROCC of 0.844, and that for miR-92a was 0.838 for CRC detection. Similarly, for advanced adenoma, the AUROCC was 0.769 for miR-29a and 0.749 for miR-92a. When used in combination, there was improved detection of CRC (sensitivity of 83 %, specificity of 84.7 %, AUROCC of 0.883) and for advanced adenomas (sensitivity of 73 %, specificity of 79.7 %, AUROCC of 0.773). Subsequent study by this group revealed

that both miRNAs are elevated in circulation of CRC patients, and decrease following surgery, and miR-92a could discriminate CRC patients from patients with gastric cancer and IBD. Additionally, these miRNAs as a panel could discriminate advanced colonic adenomas from healthy controls (sensitivity of 73 %, specificity of 80 %, and AUROCC of 0.773), suggestive of their use as early detection biomarkers [44]. Wang et al. focused on metastatic and nonmetastatic CRC patients using age and sex-matched cohorts [45]. Serum miR-29a emerged as a significant biomarker, being elevated significantly in metastatic CRC compared to patients with localized CRC, and with advanced disease prediction sensitivity and specificity of 75 % (AUROCC of 0.803). Of nine differentially expressed miRNAs in serum from stage III patients and controls, miR-18a and miR-29a showed significant elevation between cancer patients and controls [46]. Faltejskova et al. were critical of miR-17-3p, miR-29a, miR-92a, and miR-135b as circulating CRC biomarkers [47]. Serum levels of the first three did not differ between cancer and controls, and miR-135b levels were too low to be assessed properly. But the authors agree that miR-29a levels correlate with clinical stage. Further studies are warranted to establish their roles in CRC.

Several other circulating miRNAs of diagnostic relevance of CRC have been reported. Of 22 miRNAs deregulated in plasma from patients with CRCs and advanced adenomas compared to healthy controls, miR-601 and miR-760 were significantly decreased with diagnostic potential [48]. Collectively, the two had a sensitivity of 83.3 % and specificity of 69.1 % for CRC detection (AUROCC was 0.792) and a sensitivity and specificity of 72.1 % and 62.1 %, respectively, for advanced adenoma (AUROCC was 0.683). Of nineteen (out of 380) miRNAs that were deregulated in CRC tissues, miR-31 and miR-135b were the most upregulated and miR-1 and miR-133a the most downregulated [49]. These miRNAs performed well in a test set with perfect sensitivity at a specificity of 80 %. In plasma, however, miR-21 differentiated CRC patients from controls at sensitivity and specificity of 90 %.

A study aimed at identification of CRC-specific miRNAs in circulation that included samples from breast, prostate, and renal cancers, and melanoma patients, identified miR-34a as being reduced in CRC and breast cancer samples [50]. Additionally, of seven miRNAs that were differentially expressed between CRC and control tissues and blood samples, miR-23a, miR-193a-3p, and miR-338-5p were significantly increased and were more associated with advanced stage disease [51]. These three miRNAs could detect CRC at a sensitivity of 80 % and specificity of 84.4 % with AUROCC of 0.887. Another set of nine miRNAs (miR-18a, miR-20a, miR-21, miR-29a, miR-92a, miR-106b, miR-133a, miR-143, miR-145) were differentially expressed in plasma from CRC patients and controls [52]. Their levels achieved a diagnostic AUROCC of 0.708 but could not differentiate advanced adenomas from controls. MiR-15b, miR-19a, miR-19b, MiR-29a, and miR-335 are other potential CRC diagnostic circulating miRNAs [53]. Another group found the levels of miR-15b, miR-18a, miR-19a, miR-19b, miR-29a, and miR-335 to be elevated in plasma from CRC patients compared to controls. As diagnostic biomarkers for the detection of CRC, the AUROCC for these miRNAs

ranged from 0.70 to 0.80. Of interest, miR-18a was significantly increased in plasma from patients with advanced adenomas compared to healthy controls (AUROCC was 0.64). Kanaan et al. initially examined five deregulated miRNAs in plasma from CRC patients [54]. They subsequently focused on the identification of screening biomarkers for adenomas and early CRCs by applying a microfluidic approach that enabled identification of eight mRNAs (miR-15b, miR-17, miR-142-3p, miR-195, miR-331, miR-532, miR-652) in plasma for detection of polyps with AUROCCC of 0.868. However, three miRNAs (miR-15b, miR-139-3p, and miR-431) could distinguish late stage IV cancers from controls with AUROCC of 0.896. For differentiating CRC from controls and people with polyps, these three miRNAs had performances of AUROCC of 0.829 (from healthy controls) and 0.856 (from polyps).

Frequently studied circulating miRNAs with diagnostic potential for CRC include miR-18, miR-21, miR-29, and miR-92a. The diagnostic role of these miRNAs warrants further investigation.

7.4.5.2 Circulating CRC Prognostic miRNA Biomarkers

A number of circulating miRNAs in CRC patients are of potential prognostic relevance. Pu et al. used qPCR to detect differential miRNA levels in plasma without RNA extraction [55]. Of three miRNAs (miR-21, miR-221, and miR-222), miR-221 emerged as a biomarker for CRC detection. The elevated levels were significantly associated with poor OS (HR, 3.478) and correlated with p53 protein detection by IHC. Cheng et al. assayed serum miR-21, miR-92, and miR-141, and observed that miR-141 correlated with TNM stage (was more frequently associated with stage IV CRC than other stages) [56]. The levels of this miRNA had a sensitivity of 77.1 %, specificity of 89.7 %, and AUROCC of 0.836 in differentiating CRC liver metastasis from other stages (I–III). An elevated serum level predicted poor survival and was an independent prognostic factor for advanced CRC. Furthermore, it enhanced the accuracy of CEA in CRC detection. Serum miR-29a levels are significantly higher in CRC with liver metastasis than in non-liver metastatic disease patients.

Toiyama et al. designed a three-phase study that enabled the discovery of miRNAs associated with late stage metastatic disease and identified miR-200b, miR-200c, miR-141, and miR-429 as relevant [57]. Following validation, their expression was examined in tissues from patients with localized CRC and those with liver metastasis. Serum miR-200c was significantly elevated in stage IV compared to stage III CRC. These elevated levels were associated with advanced stage disease, and high miR-200c was an independent predictor of lymph node metastasis (HR 4.81), tumor recurrence (HR 4.51), and adverse prognosis (HR 2.67). Low levels of circulating miR-21 are associated with higher local recurrence and mortality [58]. Thus, relative increases in miR-21 were associated with 51 % reduction in risk of recurrence, and 50 % reductions in risk of death, and were an independent predictor of survival. Zanuto et al. examined miRNAs

differentially expressed between CRC and people with normal colonic tissues using plasma samples and subsequently validated in independent samples. Four miRNAs were different in samples from patients and controls. MiR-21 and miR-378 were validated in plasma. However, miR-378 levels decreased after surgery in non-relapsed patients 4–6 months following surgery. MiR-29c was uncovered as recurrence predictor, and the serum levels were then assessed for noninvasive applications [59]. MiR-29c is a tumor suppressor, such that high levels inhibit cancer cell proliferation, growth, and migration. Preoperative circulating miR-29c significantly decreased during early relapse compared to non-relapsed stage II/III CRC patients. Shivapurkar et al. validated circulating levels of miR-15a, miR-103, miR-148, miR-320a, miR-451, and miR-596 for use in predicting early CRC recurrence following surgery [60]. Hierarchical clustering and Kaplan–Meier analysis revealed these miRNAs could predict recurrences of early CRC with HR of 5.4 ($p = 0.00026$). High serum miR-27b, miR-148a, and miR-326 were associated with reduced PFS in mCRC patients (individual HRs were 1.4 $p = 0.004$, 1.3 $p = 0.007$, and 1.4 $p = 0.008$, respectively) [61]. MiR-326 was also associated with reduced OS (HR 1.5 $p = 0.003$).

While all studied miRNAs hold prognostic promise for CRC, the most consistent miRNAs are miR-21, miR-141, and miR-148.

7.4.5.3 Circulating CRC Predictive miRNA Biomarkers

A few circulating miRNAs have demonstrated predictive ability in CRC patients. Kjersem et al. examined predictive miRNA for mCRC patients who were to receive first-line 5FU and oxaliplatin treatment [61]. In nonresponders, three miRNAs (miR-106a, miR-130b, and miR-484) that are associated with inflammatory bowel disease were elevated in serum samples prior to treatment. The possible cardiotoxicity monitoring using miRNA in circulation of CRC patients on bevacizumab was addressed [62]. Five miRNAs in circulation were significantly increased in patients who developed cardiotoxicity compared to those without cardiac defect. But miR-1254 and miR-579 were more specific in validation studies and could serve as biomarkers for bevacizumab cardiotoxicity in CRC patients. Zhang et al. examined the role of circulating miRNAs in predicting patient response to chemotherapy [63]. Of 17 informative serum miRNAs, five (miR-21a, miR-130, miR-145, miR-216, and miR-372) were validated as chemosensitivity predictive biomarkers with AUROCC of 0.841.

7.4.6 Circulating CRC Protein Biomarkers

7.4.6.1 Traditional Serum CRC Protein Biomarkers

Several CRC protein biomarkers identified by ELISA, RIA, and other traditional assays in tissues, cell lines, and body fluids are available. Some are validated and recommended for clinical use. Serum CRC biomarkers encompass antigens (e.g., CEA and CAs), antibodies, and cytokines. However, other proteins outside these three major classes have been tested for CRC detection. These include sialylated Lewis antigen, SEX and CO29-11 (secreted by colon and other cancers), PA8-15 (a tumor-associated antigen), small intestinal mucin antigen (SIMA), u-PA, PSA, and TPA-M. Many of these do not reach the required accuracy as a screening biomarker, usually having sensitivities below 50 %. TPA-M with sensitivity and specificity of 70 % and 96 %, and u-PA of 76 % and 80 % are promising antigens among them.

Circulating CEA as CRC Biomarker

Serum CEA levels have been extensively evaluated for use in CRC screening, prognosis, postoperative surveillance, and treatment monitoring in advanced stage disease. This is the first reported blood biomarker for CRC detection. However, sensitivities have ranged from 43 to 69 % and are stage dependent, increasing with advanced stage. For example, 8 % of patients with Duke A and up to 89 % of Duke D patients are detected using this assay. Specificity is however as high as 95 %. These variable performances especially in sensitivity are also attributable to the different cutoff values used for disease detection. For instance, a cutoff at 3 ng/ml is associated with lower specificities. In many studies, however, cutoff values between 2.4 and 10 ng/ml have been used to determine diagnostic performances. CEA is not recommended for screening of asymptomatic population for early CRC detection due to the unacceptable low sensitivity and specificity. However, it is useful for predicting patient outcome following diagnosis, postoperative surveillance, and treatment monitoring in advanced stage disease.

Preoperative serum levels of CEA have proven useful as prognostic biomarkers independent of clinical disease stage. Measurement of serum CEA is recommended as an adjunct to other clinicopathologic findings in making surgical decisions. Thus, the College of American Pathologists (CAP) includes preoperative CEA in their category 1 prognostic factors for CRC. This means CEA is "definitely proven to be of prognostic importance based on evidence from multiple statistically robust published trials and generally used in patient management". Hence, preoperative CEA levels >5 ug/l are clinically used to select patients requiring further evaluations to detect distant metastasis. However, it has limited use in making decisions on adjuvant therapies.

Various primary studies and meta-analytical studies of CEA indicate that it provides useful information for monitoring CRC patients requiring surgery. In this setting, serial measurements are conducted 3 monthly apart over a period of 3 years in stage II and III disease patients who undergo surgery or systemic

treatment for metastatic CRC. While no standardized guidelines exist, CEA levels above 30 % of the previous value are clinically significant. When confirmed with another sample taken within a month, the patient is evaluated further for the possible presence of recurrent or metastatic disease. Persistently low levels (below the 30 % level) may still warrant further investigation, especially if there is other clinical evidence of possible recurrence. Indeed, some consider increases of between 15 and 20 % over three successive assays to be sufficiently informative of disease recurrence. CEA measurements are equally useful in treatment monitoring of patients with advanced stage CRC. There certainly is a clinical and an economic need to monitor for therapy efficacy, effectiveness, and possible toxicity in advanced stage CRC patients on systemic treatment. CEA measurements are conducted every 1–3 months in such patients. When levels remain persistently high (>30 %), this often suggests ineffective treatment or disease progression. It should however be noted that in the early stages of treatment, some chemotherapeutic agents (e.g., 5-fluorouracil and levamisole) can cause CEA release independent of disease activity.

The value of CEA in accurately detecting recurrences following curative intent surgery has been evaluated by systematic review [64]. Forty-two studies involving 9834 CEA measurements with long follow-up outcomes were included in the analysis. In general, the sensitivity ranged from 50 to 80 % with specificity and NPV of >80 %. However, the PPV was unreliable. This analysis did not find CEA useful in early detection of treatable CRC recurrences.

Circulating CA as CRC Biomarker

Serum carbohydrate antigen (CA) levels are also associated with CRC. Many studies have assessed the utility of CA19-9 in CRC detection. Using a cutoff value of 37 units/ml, reported sensitivities have ranged from 18 to 65 % with specificities of above 90 %. The cause for the range in sensitivity is similar to those of CEA, being stage dependent with 0 % reported for Duke A and >50 % for Duke D. Higher detection rate is observed in advanced stage III/IV disease. Similar performances are reported for the other CAs (e.g., CA242).

Circulating TIMP1 as CRC Biomarker

Tissue inhibitor of matrix metalloproteinase 1 (TIMP1) has potential for screening and prognostication of CRC. TIMP1 is a 25 kDa glycoprotein that inhibits matrix metalloproteinases, enhances cell proliferation, and inhibits apoptosis to promote tumor growth and progression. Total TIMP1 (free and those in complexes with MMPs) levels in circulation are significantly higher in CRC patients than controls, which include healthy volunteers, patients with colorectal adenomas, IBD, and even breast cancer. For detection of early colon cancer, TIMP1 outperforms CEA but has similar sensitivity for detection of rectal cancers. Preoperative plasma levels are prognostic factors independent of stage and tumor location. In stage II CRC, survival benefits of patients with low plasma TIMP1 levels were similar to age- and sex-matched background population.

7.4.6.2 The CRC Secretome

Cancer cell-conditioned medium (secretome) leads to identification of secreted circulating biomarkers. A few studies have involved CRC cell lines with the discovery of a number of possible CRC circulating proteins and peptides. Wu et al. used a panel of 21 cancer cell lines representing 12 types of tumors to screen for circulating biomarkers [65]. Three hundred and twenty five (325) unique secretome proteins were identified. Collapsin response mediator protein 2 (CRMP2) was selected as a CRC biomarker that was validated by IHC and was expressed by 58.6 % of the tumors. Plasma levels were higher in CRC patients compared to controls with sensitivity of 87 % and specificity of 95 %. A follow-up study involved 23 cell lines of 11 cancers including three CRC cell lines (COLO 205, SW620, and SW480). Several (4584) proteins were identified by LC-MS/MS of which only 30 % uniquely represented one cancer type (the majority overlap in many cancers). One hundred and nine (109) proteins were unique to CRC and are considered promising CRC proteome biomarkers. Other potential serum CRC biomarker from secretome analysis is tumor-associated antigen 90 (TAA90K/Mac-2BP) [66]. Plasma levels were significantly higher in CRC patients ($N = 280$) than healthy controls and enhanced the performance of CEA in CRC detection. Xue et al. also used secretome analysis of primary CRC cell line SW480 and metastatic CRC cell line SW620, which enabled identification of 145 altered proteins [67]. Trefoil factor 3 (TFF3) and growth and differentiation factor 15 (GDF15) were unregulated and validated in tissue and serum samples to have predictive potential for mCRC. GDF15 could differentiate CRC patient sera from controls at a sensitivity of 53.3 % and specificity of 99.4 %, while TFF3 performed at a sensitivity of 77.8 % and specificity of 97.4 %.

7.4.6.3 Serum Protein and Spectral Peaks as CRC Biomarkers

Several MS studies have used protein peaks in circulating samples and classification algorithms for CRC detection. Additionally, some identified peptides and proteins have provided encouraging performances for CRC detection. While none is a valid biomarker at the moment, they are of relevance in the pursuit for the simple noninvasive CRC screening biomarker. A few illustrative examples including human neutrophil peptides 1, 2, and 3 (HNP 1–3), also known as alpha defensins 1, 2, and 3, are provided herein.

Serum human neutrophil peptides (also known as α-defensins) have been identified as CRC biomarkers. Tissue CRC biomarkers were successfully analyzed in serum samples, leading to the identification of α-defensins as low-abundant CRC-derived biomarkers. [68]. Albrethsen et al. also compared serum biomarkers to tissue profiles and encountered α-defensins 1, 2, and 3 as CRC biomarkers [69]. While promising, serum levels of α-defensin are also elevated in infectious states and hence may reduce their specificity for CRC.

Chen et al. detected four serum peaks with *m/z* 4476, 5911, 8930, and 8817 to be unique to CRC [70]. When used in artificial neural network, these biomarkers achieved a sensitivity of 87 % and specificity of 79 % in validation data set for CRC detection. Serum proteomic profile between CRC patients and age- and sex-matched healthy volunteers was assessed with IMAC chip [71]. The most accurate classifier included peaks with *m/z* 4002 and 8132, which achieved a similar sensitivity and specificity of 95 % in validation studies. Ward et al. compared serum proteomic patterns of CRC patients and controls composed of healthy people and those with diverticular diseases [72]. Classification algorithm using the seven most informative peaks achieved a sensitivity and specificity of 94 % and 96 %, respectively. CRC proteins identified included C3a des-Arg, α-1 antitrypsin, and transferrin. Zheng et al. identified four peaks with *m/z* 3191.5, 3262.9, 3396.3, and 5334.4 that performed at a sensitivity and specificity of 90.3 % and 95.7 %, respectively [73]. Validation studies maintained such high performance with a sensitivity of 87.5 % and specificity of 93.8 %, which are much superior to CEA, CA19-1, and CA242. Proteomics of sera from discovery and validation cohorts of CRC patients and controls, coupled with diagnostic model development based on class comparison and custom algorithm, enabled the confirmation of C3a des-Arg (the stable form of C3a anaphylatoxin) as elevated in CRC. An ELISA validation of C3a des-Arg achieved a sensitivity of 96 % for CRC and 70 % for colorectal adenoma detection. Engwegen et al. used two independent sample sets to discover informative peaks and their diagnostic potential determined using a classification tree [74, 75]. The sensitivity and specificity of these classification models were between 65 and 90 %. The peaks were identified as N-terminal fragment of albumin, apolipoprotein C-I, and apolipoprotein A-I. Apo C-I was validated in a prospective study of patients with CRC, adenomatous polyps, and healthy controls. Class prediction performed at 58 %, but the authors suggest this could improve with the use of quantitative assay with cutoff values. CRC-specific protein peaks were sought with controls inclusive of people with other malignancies, patients with noncancerous conditions, and healthy volunteers [76]. This SELDI-TOF MS whole serum proteomic profiling was not specific to CRC but could differentiate cancerous from noncancerous samples. In another series, four discriminating peaks from a training set achieved a sensitivity and specificity of 79 % and 71 %, respectively, in a blind validation set, but a three-peak diagnostic model could stratify CRC patients, patients with benign colorectal conditions, and healthy controls [77]. However, these peaks were not validated.

Some of these peptides including C3a des-Arg, Apo CI, HNP1, HNP2, and HNP3 are of interest because more than a single study has detected them.

7.4.7 Circulating CRC Metabolomic Biomarkers

Serum metabolites have been explored for the diagnosis, prognosis, and staging of CRC patients. Nishiumi et al. used GC–MS to identify differentially expressed

serum metabolites between CRC patients and controls [78]. A CRC predictive model that included 2-hydroxybutyrate, aspartic acid, kynurenine, and cystamine was developed for CRC detection. In test data set, sensitivity, specificity, and accuracy were 85 % each, with AUROCC of 0.9097. The accuracy was much superior to CEA and CA19-9 in this series. The performance of this CRC metabolic predictive model was equally impressive in a validation data set with a sensitivity, specificity, and accuracy of 83.1 %, 81 %, and 82 %, respectively. Of interest, early stage (stage 0–II) CRCs could be detected at a sensitivity of 82.9 %. Ritchie et al. obtained pretreatment sera from CRC patients in the United States and Japan for metabolomic biomarker discovery using Fournier transfer ion cyclotron resonance MS (FTICR-MS), followed by structural characterization by LC MS/MS and NMR [79]. The clinical translational ability was also demonstrated using high-throughput triple-quadrupole multiple reaction monitoring (TQ-MRM). Significantly reduced circulating levels of 28–36 carbon-containing hydroxylated polyunsaturated ultra-long-chain fatty acids were demonstrated in three independent cohorts of CRC patients compared to healthy controls. The TQ-MRM method based on three metabolite biomarkers validated the results in two further independent studies achieving an AUROCC of 0.85–0.98 (mean 0.91 ± 0.04). Ma et al. used GC–MS to monitor metabolic changes in CRC patients before and after surgery [80]. In the small sample of 30 patients, 34 endogenous metabolites were identified that discriminated between pre- and post-operative patients. Postoperative state was associated with decreased circulating levels of L-valine, 5-oxo-L-proline, 1-deoxyglucose, D-turanose, D-maltose, arachidonic acid, and hexadecanoic acid, with increases in the levels of L-tyrosine. In a follow-up study, six of the 34 metabolites could differentiate 93.5 % of CRC patients from healthy controls using supervised predictive models [81]. The application of GC–TOF MS and ultraperformance liquid chromatography-quadrupole time-of-flight MS (UPLC–QTOFMS) analysis of sera from CRC patients and healthy controls identified significantly discriminatory metabolites. Of 33 differentially circulating metabolites, five were assayed by both techniques. Possible deregulated metabolic pathways in these patients include glucose, arginine, proline, fatty acid, and oleamide. The distinct CRC metabolites are involved in TCA and urea cycles, fatty acid, glutamine metabolism, and gut microbiota [82].

A circulating prognostic metabolomic signature was sought using 1H NMR to profile sera from 153 patients with mCRC and 139 healthy controls [83]. In a training set, metabolite profile could differentiate all mCRC patients from controls, and the accuracy was equally strong at 96.7 % in the validation cohort. An overall survival predictor generated from the training data set could detect patients with significantly reduced survival outcome (HR, 3.4; 95 % CI 2.06–5.50; $p = 1.33 \times 10^{-6}$). Sera from 103 CRC patients with various degrees of metastasis were subjected to both 1H NMR spectroscopy and GC–MS [84]. Serum metabolite profile could significantly distinguish between patients with locoregional metastasis and those with liver involvement, as well as between patients with liver metastasis and those with distant metastasis. These biomarkers can enhance staging that currently depends on radiographic and pathologic evaluation with their known limitations.

7.4.8 Circulating CRC Cells

Despite improved detection, ~25 % of CRC patients present with liver metastasis, and ~50 % will eventually develop metastatic disease. Thus, circulating colorectal cancer cell (CCRCC) characterization is important in optimal disease management. This has been assessed in the nonmetastatic and metastatic settings.

7.4.8.1 CCRCCs in Patients with Nonmetastatic CRC

Circulating colorectal cancer cells in nonmetastatic cancer patients have been detected at low frequencies, and their prognostic role has mixed results in many small studies. However, a large study targeting the expression of *KRT19*, *KRT20*, *CEA*, and *hTERT* in samples from 194 patients with stage II disease provided possible value of evaluating CCRCCs in early stage disease. This study identified depth of tumor invasion, vascular invasion, and expression of CCRCC markers as prognostic biomarkers in stage II disease. The presence of any one of these predictive parameters conferred 27-fold hazard ratio of postoperative relapse [85]. A meta-analysis involving 646 patients concluded that CCRCC detection in the portal vein correlated with nodal invasion, and CCRCC status at surgery is associated with disease-free survival and liver metastatic relapse independent of tumor stage [86]. Another comprehensive meta-analysis of 36 studies involving 3094 nonmetastatic CRC patients examined the prognostic value of both disseminated CRC cells and CCRCCs in the portal vein and systemic circulation. Pooled studies indicated initiating tumor cell (ITC) detection was significantly associated with shorter recurrent free intervals and OS. With regard to the anatomic sites of ITCs, only cells in the systemic circulation were of prognostic relevance. Disseminated tumor cells and portal vein CCRCCs failed to reach significance for overall survival [87].

7.4.8.2 CCRCCs in Patients with Metastatic Disease

CCRCCs in patients with metastatic disease provide useful prognostic information and can also be characterized for genetic alterations.

Disseminated tumor cells have failed to be of any prognostic use in CRC. However, CCRCC characterization either by molecular or cytologic methods has clinical value. The CELLSEARCH® system of CTC characterization is approved for monitoring metastatic CRC. This FDA approval was achieved after the study by Cohen et al. that provided strong evidence for the independent prognostic value of CCRCC enumeration in patients [88]. Baseline CCRCCs $\geq$ 3/7.5 ml of blood were associated with worse PFS and OS. Subsequent analysis of the CCRCC-positive cohort revealed that those who converted from CCRCC positive to negative at 3–5 weeks following chemotherapy had better PFS [89]. Tol et al. observed similar

findings as Cohen et al., demonstrating that mCRC patients with ≥3CTCs/7.5 mls had worse PFS (8 vs. 10.5 months) and OS (14 vs. 22 months) [90]. However, on follow-up, those who converted to CCRCC negative at 1–2 weeks (not 3–5 weeks as demonstrated by Cohen) had better outcomes. There is a need to resolve the disparity as to when to resample to assess survival benefit from chemotherapy. The little difference in the studies could be explained by the low detection rate of CCRCCs at 3–5 weeks in the Tol et al. study (5 %) compared to the 12 % by Cohen et al. Reportedly, the use of bevacizumab, as in this study, reduces CTC detection, at least in breast cancer patients.

A meta-analysis performed using 12 studies inclusive of 1329 patients with mCRC indicated that both OS (HR, 2.47) and PFS (HR, 2.07) were worse in patients positive for CCRCCs. Indeed, in eight of the studies, multivariate analyses were performed, and all reached similar conclusion that CCRCC is an independent prognostic predictor of survival [91].

About ~15 % of CRC metastatic liver deposits can be resected with curative intent. These patients often receive intensive polychemotherapy with or without targeted therapies to shrink the tumor before surgery. In other to select patients who will benefit from these radical procedures with regard to achieving longer PFS, surgeons have focused on examining CCRCCs [92, 93]. Also of surgical concern was the finding that CCRCC release during radio-frequency ablation of liver metastasis was much higher than during surgical resection [94, 95].

Genetic analysis has been performed on CCRCCs as a noninvasive means of disease monitoring and for making treatment decisions. Mutations in *KRAS*, *BRAF*, *PIK3CA*, and *EGFR* have shown promise as novel biomarkers that can be sampled even in single CCRCCs. *KRAS* mutations were detectable in tumors and CCRCCs at a frequency of 43.4 % and 39.5 %, respectively, and these were highly correlated. Many patients who responded to cetuximab plus chemotherapy had wild-type *KRAS* in both tissues and CCRCCs, and this was associated with better OS and PFS [96]. Gasch et al. demonstrated molecular characterization of single CCRCCs [97]. They observed heterogeneous expression of *EGFR* and mutations in *KRAS*, *EGFR*, and *PIK3CA* in the same patient or between patients, which possibly underlie the heterogeneous response to anti-EGFR therapy. Others have found discordant mutation status and patterns between primary tumor, metastatic deposits, and CCRCCs, which may reflect tumor evolution. Mostert et al. show a 23 % and 7 % discordances in *KRAS* and *BRAF* mutations between primary and metastatic CRC [98]. Fabbri et al. also demonstrated discordant mutations in *KRAS* between primary tumor and CCRCCs [99]. Concordance was in only 50 % of matched samples. In another study, 46 % of patients had discordant *KRAS* mutations between prior tissue biopsy and CCRCCs [100]. It is also demonstrated that the mutational status of genes can change with treatment [101]. However, sensitive technologies appear to indicate increase concordance between tumor and CCRCC mutations. Mohamed et al. demonstrated 84.1 % and 90.9 % concordances of *KRAS* and *BRAF* mutations, respectively, between tumor and CCRCCs [102].

KRAS mutations, which are common in patients with CRC, are used to select patients for anti-EGFR treatment. ASCO's recommendation based on systematic

literature review is that "… *all patients with metastatic colorectal carcinoma who are candidates for anti-EGFR antibody therapy should have their tumor tested for KRAS mutations in a CLIA-accredited laboratory. If KRAS mutation in codon 12 or 13 is detected, the patients with metastatic colorectal carcinoma should not receive anti-EGFR antibody therapy as part of their treatment*" [103]. The importance of serial sampling to monitor *KRAS* and other gene mutation status, which may change as a result of tumor evolution with metastasis, is thus noteworthy.

7.5 Summary

- Colorectal cancer is one of the most commonly diagnosed epithelial cancers.
- Early detection improves the 5-year survival, which is currently being realized due to active screening coupled with improved disease management.
- Many CRCs are sporadic, with a few being of hereditary nature.
- Multiple genetic alterations occur in a defined stepwise fashion to drive CRC progression.
- There are established genes altered in CRC, which are currently being clinically applied in disease detection and management.
- The molecular genetic biomarkers of CRC are explored noninvasively as well (in circulation and stool) for CRC screening and disease management.
- Methylation (e.g., *CDKN2A* and *MLH1*) and mutations (e.g., *KRAS* and *APC*) in CRC are easily detected in body fluids (ctDNA).
- There are a number of commercially available blood tests for CRC; however, the FDA-approved test for CRC screening is methylation in *SEPT9*.
- Novel circulating CRC biomarkers include alterations in *hTERT* transcript, ncRNA, and metabolomic profiles.
- Proteomic signatures, and identified proteins in circulation, are promising biomarkers.
- Circulating CRC cells are clinically established prognostic indices in metastatic CRC and hence the approval of the CELLSEARCH® system for such utility.
- Captured CCRCCs can also be characterized for genetic alterations to identify possible novel mutant clones for targeted therapy.

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Chapter 8
Hepatocellular Carcinoma Biomarkers in Circulation

Key Topics

- Molecular pathology of hepatocellular carcinoma (HCC)
- Circulating cell-free nucleic acids as HCC biomarkers
- Circulating HCC epigenetic biomarkers
- Circulating HCC genetic biomarkers
- Circulating HCC miRNA biomarkers
- Circulating HCC protein biomarkers
- Circulating HCC cells

Key Points

- HCC is the most common primary liver cancer. It is endemic in Asia and Africa due to increased prevalence of established risk factors in these geographic regions.
- Serum biomarkers including AFP and DCP levels have proven useful in disease management, especially in prognostication and staging. Panel biomarkers hold potential for screening of the entire population at risk.
- Alterations in HCC epigenome (e.g., methylated *CDKN2A* and *CDH1*), genome (e.g., *TP53* mutations), miRNA (e.g., miR-122), as well as CTCs are detectable in circulation. The clinical potential of these circulating HCC biomarkers is being evaluated.

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8.1 Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer. Globally, HCC ranks fifth in incidence and is a major cause of cancer-related deaths. In 2012, 782,000 new cases were estimated with 745,000 case fatalities in the world. Similar dismal outcome statistics apply to the US where 39,230 new cases and 27,170 deaths are expected in 2016. Thus, similar to lung cancer, the incidence mirrors the mortality rates, suggestive of the need for early detection and effective treatment. Validated biomarkers in body fluids should help revert this dismal statistics. The majority of HCCs (~80 %) occurs in Eastern and Southeastern Asia (where over half of the cases are diagnosed) and sub-Saharan Africa, due to the increased prevalence of known risk factors in these regions. The incidence is equally rising in the Western world due to the increasing incidences of hepatitis C viral (HCV) infections. The established risk factors for HCC include chronic hepatitis B and C viral infections, cirrhosis of any cause, and high-level exposure to aflatoxin-b1 (AFB1). Additional factors that modestly elevate the risk include diabetes, nonalcoholic fatty liver disease, obesity, and smoking.

Liver resection and transplantation are useful treatment strategies for tumors detected early, while advanced stage disease patients are subjected to chemotherapy and other targeted therapies. The 5-year survival rate for patients with HCC in general is ~40 %, but when detected early, this can be as high as 60–70 %. Hence the need for screening programs, especially in endemic areas. Imaging modalities (US, CT, MRI) are generally used in the Western world for HCC screening. However, serum biomarkers that offer a cost-effective means of early detection are the screening methods of choice in Asia. But the alpha-fetoprotein (AFP) serum biomarker for HCC suffers from sensitivity and specificity. Even when combined with des-γ-carboxyprothrombin (DCP) in a panel, sensitivity ranges from 48 to 94 % with equally variable specificities of 53–99 % for early HCC detection. The need for more accurate early detection biomarkers in body fluids is obvious and being pursued.

8.2 Screening Recommendations for HCC

Screening for HCC is not recommended for the general population, even in regions such as Asia with higher incidences (up to 80/100,000 in China) than other parts of the world. The NCI notices that screening of people at elevated risk does not reduce mortality and also observes that invasive procedural screening such as needle aspiration cytology may carry risks such as bleeding, pneumothorax, and bile peritonitis. Surveillance is however recommended for people with HBV infection or carriers, HIV coinfection, or cirrhosis of any cause. Surveillance is not recommended for HCV-infected people except in the presence of cirrhosis or coinfection with HBV or HIV. For this population at elevated risk above average, 6–12 monthly abdominal ultrasound and serum AFP assay are a cost-effective means of early cancer detection.

8.3 Molecular Pathology of HCC

Primary liver cancer can originate from any cellular components of the liver. However, HCCs, which are cancers of hepatocyte origin, constitute 85–90 % of all liver cancers. The next in frequency are intrahepatic cholangiocarcinomas (5–10 %) that arise from the cholangiocytes lining bile ductules in the liver. Constituting <5 % of cancers of the liver are hepatocellular adenomas, hepatoblastomas, epithelioid hemangioendotheliomas, and fibrolamellar hepatocellular angiosarcomas. The best-studied molecular pathology pertains to primary HCC.

The risk factors of HCC implicate an inflammatory process in its pathogenesis. Consistent with inflammatory-mediated diseases, reactive oxygen and nitrous species, as well as cytokine excess can damage hepatocytes with subsequent regeneration. Chronically damaging events to cells can result in epigenetic and genetic alterations leading to oncogene induction and/or tumor suppressor gene inactivation. These events in turn can impinge upon at least four important signaling pathways (WNT, TGFβ, PI3K/AKT, and RAF/MEK/ERK) implicated in HCC progression.

8.3.1 Specific Genetic and Signaling Pathway Alterations in HCC

Of importance in hepatocellular carcinogenesis are chromosomal and microsatellite alterations. Chromosomal losses at 1p, 4q, 6q, 8p, 9p, 13q, 16q, 16p, and 17p, as well as gains at 1q, 6p, 8q and 17q, characterize HCCs. These chromosomal regions harbor tumor suppressor genes such as *RB*, *TP53*, *CDKN2A*, and *IGF2R* and oncogenes including *CTNNB1* that play important roles in the pathogenesis of HCC.

The epigenome is equally deregulated in HCC, and this is an early event in tumor progression. Altered promoter methylation, histone modifications, and miRNA expression underlie disease progression. Promoter hypomethylation and activation of oncogenes, as well as hypermethylation and silencing of tumor suppressor genes, are established in HCC, as in other cancers. *PTEN*, *RB*, and *CDKN2A* are inactivated in HCC partly via promoter hypermethylation. Also hypermethylated are *BMP4*, *SPDY1*, *DAB2IP*, *GSTP1*, *FZD7*, *ZFP41*, *APCNFATC1*, and *RASSF1A* that are established and putative tumor suppressor genes. Promoter hypermethylation of *DCC*, *CSPG2*, and *NAT2* is associated with HBV-mediated HCC. *PAX4*, *SCGB1D1*, and *WFDCG* promoters are hypomethylated in HCC. Of interest, DNA methyltransferase (DNMT) enzymes are overexpressed early in HCC development, underscoring the early gene methylation status in this cancer.

In addition to mutations, epigenetic and chromosomal alterations, liver cancer cells express and respond to several growth factors including EGF, FGF, PDGF, IGF, and VEGF that act on receptor tyrosine kinase receptors to induce various signaling pathways to sustain malignancy and promote invasiveness. Thus, growth factor signaling is associated with proliferation, invasiveness, portal thrombosis, and increased neovascularization, which is a characteristic of HCC.

8.3.1.1 RAS-MAPK Pathway Alterations in HCC

The RAS/RAF/MEK/ERK pathway plays important roles in liver cancer development. Similar to many signaling networks, this pathway is activated in HCC through diverse mechanisms. HBV protein HBx and HCV core protein can activate this pathway. Downregulation of *RKIP* and *RAS* inhibitors, *SPRED1* and *SPRED2* in HCC is associated with pathway activation as well. Besides, growth factor signaling plays a role in activating this pathway. Not surprisingly, almost all HCCs have the RAS/RAF/MEK/ERK pathway engaged, although mutations in *RAS* (except *NRAS,* which is mutated in ~30 % of liver cancers) and *RAF* are infrequent in HCC. The importance of this pathway in HCC has led to its exploration as a therapeutic target, with the pathway inhibitor, sorafenib (Nexavar®), indicated for the treatment of patients.

8.3.1.2 PI3K/AKT Pathway Alterations in HCC

The PI3K pathway is activated in HCC via multiple mechanisms. In addition to mutations in genes of this pathway and growth factor interactions with RTK receptors, inactivation of *PTEN* occurs in many HCCs. LOH at the *PTEN* locus occurs in about 40–60 % of HCCs, and *PTEN* is also silenced via promoter hypermethylation. *AKT* activation as a consequence of loss of *PTEN* function enables the acquisition of aggressive tumor phenotypes.

8.3.1.3 Alterations in Cell Cycle Regulators in HCC

TP53, *CDKN2A*, and *RB* are important regulators of the cell cycle, and they are all deregulated in subsets of HCC. Aflatoxin-b1 (AFB1) in grains and peanuts is an established mutagen of *TP53*. Specifically, this toxin causes G:C to T:A transversions at base 3 in codon 249 leading to the substitution of arginine for serine (R249S). Additionally, *TP53* is a target of viral oncoproteins, especially HBV-encoded HBx, and hence mutation rate accelerates with viral hepatitis. Promutagenic N7-deoxyguanosine adduct formation from AFB1 mediates the G: C to T:A transversion mutation. In non-AFB1-mediated HCC, *TP53* mutations occur in later stages of the disease. For example, *TP53* mutations and LOH occur in moderately to poorly differentiated HCC of non-AFB1 origin, and this is not a

feature of precursor lesions or AFB1-induced HCC. In addition to its association with aggressive tumors with poor prognosis, *TP53* R249S causes reprogramming of terminally differentiated cells into acquisition of stem cell-like phenotypes. Several other members of the cell cycle are deranged in HCC. *RB* is inactivated in about 28 % of HCCs. *CDKN2A* expression is lost in ~34 % of HCC, while overexpression of *CCND1* and *CDK4* occurs in a majority (58 %) of these cancers. Additionally, CCNE1 is a possible target of the dsRNA-binding protein and nuclear factor 90 (NF90), both of which are elevated in HCC. These changes serve to propel the cell cycle through G1/S phase transit.

8.3.1.4 WNT/β-Catenin Pathway Alterations in HCC

Aberrant WNT/β-catenin pathway activation due to altered expression of pathway members as a consequence of epigenetic modifications, mutations, and deletions plays an important role in HCC progression. Pathway ligands, receptors, and antagonists, among other players, are deregulated in HCC, and some of these alterations occur early in precursor lesions. The WNT/β-catenin pathway gene most mutated (at a frequency of ~33 %) in HCC is *CTNNB1*. In addition to activating mutations, *CTNNB1* is a target of some miRNAs. For example, miR-214 targets this gene through EZH2 to activate the WNT/β-catenin pathway in HCC. Also mutated in HCC is *AXIN*, a tumor suppressor gene that regulates the degradation of β-catenin. *AXIN* mutations occur in about 6.2 % of HCCs, and these are late events, being almost absent in precursor lesions. *APC* mutations are rare, but epigenetic silencing is an important event in HCC. *APC* loss of function leads to lack of β-catenin phosphorylation and hence its accumulation and pathway activation. Aberrant expression is also observed in the WNT/β-catenin pathway receptors, agonists, and antagonists. The WNT/β-catenin pathway receptors, *FZD3, FZD6*, and *FZD7*, as well as co-receptor, LRP6, are all upregulated in HCC. Similarly, canonical pathway members, *WNT1* and *WNT3*, are overexpressed, while noncanonical *WNT5a* and *WNT11* are downregulated in HCC. The downregulation of several WNT/β-catenin pathway antagonists including *SFRPs* (via methylation), *DKKs*, and *WIF1* is noted in HCC. Importantly, some of these alterations occur early in HCC. Another important aspect of WNT/β-catenin pathway activation is its involvement in EpCAM+ (progenitor cell-type) HCCs.

8.3.1.5 TGFβ Pathway Alterations in HCC

The TGFβ signaling pathway controls multiple cellular activities including proliferation, growth, differentiation, apoptosis, extracellular matrix remodeling, and general cellular homeostasis. The signaling output tends to be cell-type dependent. For example, signaling in fibroblast may lead to proliferation, cytokine secretion, and extracellular matrix production, while in epithelia, apoptosis, cell cycle arrest, and adhesion may occur, and yet in endothelium, cellular proliferation, growth,

migration, and morphogenesis may be triggered. The role in liver physiology and pathology may be dual and context dependent. Indeed, the TGFβ pathway mediates all stages of chronic liver pathology, from initial injury, inflammation, fibrosis, cirrhosis, to eventual development of HCC. One role of the TGFβ pathway is in controlling liver volume, by inducing cytostasis and cellular apoptosis. On the contrary, liver damage induces increased TGFβ levels leading to liver cell damage and repair. Loss of this signaling pathway, however, causes increased hepatocyte proliferation and neoplastic transformation. Thus, TGFβ performs a tumor-suppressive function during early stages of liver damage to facilitate regeneration. On the other hand, during hepatocellular carcinogenesis, there may be a switch to enhance tumor progression to invasion and metastasis. These "fluid" roles of the pathway are evidenced in the complex of its interactome, which enables switches in receptor types and deployment of other signaling pathways including JNK signaling. This complexity has made therapeutic targeting of this pathway a formidable task. Normal hepatocytes are devoid of active TGFβ, but levels are detectable with acute liver injury and increase progressively mirroring the severity of liver damage. This increase is measurable in serum and can be used to monitor or detect live disease.

8.3.2 Molecular Subtypes of HCC

Primarily based on gene expression data, molecular subtypes of HCC have been identified and proven to have various clinical implications. In 2004, Lee et al. delineated two distinct types of HCC using gene expression signatures, and these distinct groups had different prognosis [1]. The group associated with poor prognosis had increased expression of genes involved in cell proliferation (*PCNA*, *CDK4*, *CCNB1*, *CCNA2*, and *CKS2*), prevention of apoptosis (*PTMA*/*ProT*), epigenetic histone modifications (*HRMT1L2*, which encodes a histone H4-specific methyltransferase), ubiquitination (*UBE2D*), and resistance to hypoxic conditions (*HIF1α*). This group was also enriched for AFP-positive tumors that already confer poor survival outcomes.

Another gene expression profiling placed all HCCs into six subgroups. However, based on chromosomal alterations, these six subgroups could indeed be subclassified into just two distinct categories. The first group (1–3) exhibits chromosomal instability, while the second group (4–6) harbors mostly normal chromosomes [2]. The characteristics of the first group are absence of *TP53* mutations and presence of *AXIN1* mutations in association with increased expression of imprinted genes. Moreover, this group of tumors is associated with low copy number HBV infections. The second group has increased LOH, presence of *TP53* and *AXIN1* mutations, as well as high copy number HBV infections.

Yamashita et al. also dichotomized HCCs based on expression of EpCAM and its related genes, as well as hepatic stem cell marker expressions [3]. The EpCAM-negative tumors phenotypically resembled mature hepatocytes. However, tumors

positive for EpCAM were of hepatic progenitor cell type with possible tumor-initiating abilities. Additionally, EpCAM-positive cancer cells expressed stem cell markers such as C-KIT and CK19 and had WNT/β-catenin pathway activation. Incorporating the presence or absence of AFP enabled the subclassification of these tumors into four groups with prognostic implications. The EpCAM-positive/AFP-negative (type A) tumors were associated with good prognosis; EpCAM-negative/AFP-negative (type D) tumors were of intermediate prognosis, while the worse prognosis was among patients with EpCAM-positive/AFP-positive (type B) and EpCAM-negative/AFP-positive (type C) tumors.

8.4 Circulating HCC Biomarkers

Circulating biomarkers of HCC are continuously being explored. While traditional serum biomarkers remain the mainstay of HCC detection and management, the potential of detecting tumor-specific epigenetic and genetic changes in ctDNA cannot be overlooked. Thus efforts are directed toward uncovering the clinical utility of these biomarkers. Additionally, the promise of miRNA, and the prognostic roles of circulating HCC cells need coordinated validation work.

8.4.1 Circulating Cell-Free Nucleic Acid Content as HCC Biomarkers

Circulating cell-free DNA (ccfDNA) is elevated in patients with HCC, and this may have diagnostic, staging, and prognostic utility. Using a genetic approach, Jiang et al. demonstrated elevated ccfDNA in HCC patients compared to controls [4]. Cancer patients harbored higher levels of shorter DNA molecules that preferentially carried tumor-associated copy number aberrations. Circulating mtDNA was also elevated in HCC patients, and these molecules were much shorter than circulating cell-free nuclear DNA. CcfDNA was elevated in 56.4 % of HCC patients compared to 4.4 % of healthy controls [5]. In another series, ccfDNA levels did not correlate with AFP and L-fucosidase, and the combination of the three biomarkers elevated the sensitivity from 56.4 to 89.7 % for HCC detection. CcfDNA is elevated in people with benign hepatic conditions such as cirrhosis and chronic hepatitis. The diagnostic performance for differentiating HCC patients from those with benign conditions achieved a sensitivity, specificity, and AUROCC of 91 %, 43 % and 0.69, respectively. Additionally, high ccfDNA levels were associated with shorter survival [6]. Chen et al. targeted two DNA fragments (100 bp and 400 bp) in β-actin gene to determine ccfDNA levels and to develop a DII assay for HCC [7]. Elevated DNA levels significantly differed between cancer and healthy control individuals but not those with HBV infection. However, DII

was significantly higher in HCC patients than HBV-infected individuals and healthy controls and was thus of much diagnostic utility. DII was associated with tumor size and metastasis. CcfDNA (median 173 ng/ml) levels were significantly higher in cancer patients than healthy controls (median 9 ng/ml) and people with cirrhosis and chronic hepatitis (median 46 ng/ml) [8]. The AUROCC was 0.949 and 0.874, respectively, for differentiating HCC patients from healthy controls and those with benign liver conditions. At a cutoff value of 18.2 ng/ml, ccfDNA had a sensitivity and specificity of 90.2 % and 90.3 %, respectively, for HCC detection. This improved to diagnostic accuracy with AUROCC of 0.974 (95.1 % sensitivity and 94.4 % specificity) when combined with serum AFP levels. As a prognostic biomarker, elevated ccfDNA levels were associated with tumor size, intrahepatic spread, vascular invasion, and shorter survival.

A meta-analysis was performed on the quantitative (seven studies) and qualitative (15 methylation analysis) studies of ccfDNA in HCC detection. These studies included 2424 subjects of which 1280 were cancer patients [9]. The pooled sensitivity, specificity, PLR, NLR, DOR, and SAUROCC were 74.1 %, 85.1 %, 4.97, 30.4 %, 16.347, and 0.86, respectively, for the quantitative studies and 53.8 %, 94.4 %, 9.545, 49.0 %, 19.491, and 0 .87, respectively, for the qualitative methylation studies. With the addition of AFP levels, the overall performances of both quantitative and qualitative studies increased with a DOR of 106.27 and SAUROCC of 0.96. Thus, ccfDNA has potential diagnostic applications and can augment the performance of AFP in HCC surveillance in high-risk individuals.

8.4.2 Circulating HCC Epigenetic Biomarkers

Genes found methylated at various frequencies in circulation of patients with HCC include *CDKN2A* (most frequently studied), *RASSF1A*, *CDH1*, *RUNX3*, *ZFP41*, *DAB21P*, *BMP4*, and *SPDY1*. Hypomethylation of *PAX4*, *ATK3*, *CCL20*, *WFD6*, and *SCGB1D1* in circulation of HCC patients has also been demonstrated.

Wong et al. examined *CDKN2A* methylation in tissue and plasma samples from HCC patients [10]. *CDKN2A* was methylated in 73 % of tissue samples, and these methylation patterns were detected in 81 % of paired plasma and serum samples. Promoter methylation of *CDKN2B* was demonstrated in 64 % of HCC and 25 % of plasma and serum samples as well. Either *CDKN2B* or *CDKN2A* methylation was positive in 48 % of tumors and in 92 % of plasma/serum samples. All available buffy coat samples were positive for *CDKN2B* methylation, indicative of possible contribution from CTC presence. Of the patients with positive serum/plasma samples, concurrent methylations in tumors were demonstrated in 75 % of patients who had metastatic or recurrent disease. Collectively, ccfDNA and CTCs were detected in 87 % of the 92 % who had *CDKN2B* and *CDKN2A* methylation [11]. *CDKN2A* methylation in plasma/serum and blood cells collected pre-, intra-, and post-operatively from patients with HCC was studied for its diagnostic utility.

CDKN2A was methylated in 80 % of peripheral circulation (i.e., plasma/serum or blood cells) and decreased 12- to 15-fold after surgery [12]. Methylation of *CDKN2A* in sera from 23 patients with cirrhosis and 46 HCC patients was studied, and methylation was demonstrable at a frequency of 47.8 % and 17.4 % of samples from patients with HCC and cirrhosis, respectively. Of note, these methylation patterns had no association with AFP levels, indicating a possible complementary use [13].

Tan et al. studied gene methylation in serum samples to uncover tumor-specific biomarkers of multiple cancers [14]. Promoter methylations of four tumor suppressor genes (*CDKN2A*, *RASSF1A*, *CDH1*, and *RUNX3*) were demonstrated in metastatic breast cancer, NSCLC, gastric, pancreatic, colorectal, and liver cancers. Hypermethylation of at least one of these genes was found in 88.6 % of serum samples from cancer patients, with the following frequencies in the different cancers: 80 % of metastatic breast cancers, 95 % of NSCLC, 82.4 % of colorectal cancer, and all of the limited gastric, pancreatic, and liver cancer samples. This gene panel appears useful as noninvasive screening for cancer detection in general. However, the most frequently methylated (62.9 %) gene in circulation of cancer patients was *RUNX3* [14]. In a study whereby plasma samples from HCC patients were subjected to genome-wide methylation analysis, the genes frequently hypermethylated were *CDKN2A*, *ZFP41*, *DAB21P*, *BMP4*, and *SPDY1*, while *PAX4*, *ATK3*, *CCL20*, *WFD6*, and *SCGB1D1* were mostly hypomethylated [15].

Although no strong diagnostic association is made in these pilot studies, the potential of using circulating methylation biomarkers in HCC patient management exists should in-depth analysis be performed using sensitive technologies. Thus, Wen et al. developed a methylated CpG tandem amplification and sequencing (MCTA-Seq) method that could detect thousands of promoter hypermethylation simultaneously using just 7.5 pg of ccfDNA [16]. This approach enabled detection of gene methylations in plasma from patients with small tumors (≤3 cm) and even those negative for AFP serum assay. Genes of interest in HCC diagnostics detected by this method include *RUNX2*, *VIM*, *RGS10*, and *ST8SIA6*. Similarly, targeted deep methylation analysis of plasma ccfDNA using massively parallel semiconductor sequencing enabled detection of methylation in *H19*, *IGF2*, *VIM*, and *FBLN1* in cancer patients [17].

8.4.3 Circulating HCC Genetic Biomarkers

8.4.3.1 Circulating Mutated Genes as HCC Biomarkers

Mutations in *TP53* are detected in clinical samples from patients with HCC. It appears that the *TP53* codon 249 mutation is a tumor marker, and its detection in plasma from patients with liver cirrhosis predates the development of liver cancer by several months. Plasma and tissue analyses of *TP53* mutations show

concordance in over 50 % of cases, indicating its usefulness as a noninvasive biomarker for HCC.

In endemic areas such as sub-Saharan Africa and China, *TP53* ser249 mutations in plasma are strongly associated with HCC. Its frequency is lower in samples from healthy controls compared to patients with liver cirrhosis and much higher in patients with HCC [18]. Using a more sensitive method for mutation detection in plasma (short oligonucleotide mass analysis – electrospray ionizing mass spectrometry), Jackson et al. were able to detect this mutation in plasma from >50 % of patient samples [19]. Four plasma samples in this series that tested positive for mutations had no associated tissue mutation (another example of tissue heterogeneity that is overcome by body fluid analysis). In another study, this mutation was found in over 46 % of post-diagnostic patient plasma samples and was demonstrable in plasma over a year prior to diagnosis in some cases, suggesting its noninvasive use in early detection of HCC, at least in endemic areas. Szymanska et al. detected this mutation at a similar high frequency of 64 % in pre-diagnostic plasma using the short oligonucleotide mass analysis, confirming this method as useful for such analysis [20]. The circulating levels has strong concordance (88.5 %) with tissue presence [21], indicative of its applicability as an authentic biomarker for patient management.

8.4.3.2 Circulating MSA as HCC Biomarkers

Loss of tumor suppressor genes (TSGs) on chromosome 8p is associated with HCC progression and metastasis. The metastasis suppressor, *HTPAP-1*, for instance, is strongly associated with HCC metastasis and prognosis [22]. Another potential TSG located on 8p22 that may be involved in hepatocellular carcinogenesis is *deleted in liver cancer 1* (*DLC1*).

An initial study of LOH using three chromosome 8 markers (D8S277, D8S298, and D8S1771) found a high concordance rate between tissue and plasma MSA. In considering all three loci, concordance was 73.3 %, indicating plasma MSAs are of HCC origin [23]. In a follow-up study, the three chromosome 8 markers were used for LOH detection in plasma from HCC patients as a biomarker of clinical disease course. LOH at one or more loci was observed in 58.1 % of plasma samples and was associated with intrahepatic metastasis but not other clinical parameters studied, including HBsAg, cirrhosis, AFP levels, tumor size, and differentiation [24]. Chromosome 8 deletions were however shown to be associated with metastasis, and whether these changes could be assayed in plasma was questioned. Other chromosome 8 markers (D8S258 and D8S264) have been used for analysis of MSA. The levels of ccfDNA in plasma were associated with tumor size, TNM stage, and poor 3-year DFS and OS. Additionally, allelic imbalance (AI) at D8S258 was associated with tumor differentiation, TNM stage, vascular invasion, and poor 3-year DFS and OS. The presence of both high circulating DNA levels and AI at D8S258 in the same patient was associated with an even much dismal 3-year survival outcome [25].

8.4.4 Circulating HCC Coding RNA Biomarkers

HTERT and TGFβ1 transcripts have been investigated as circulating biomarkers of HCC. Miura et al. aimed at identifying a biomarker for early detection of HCC that could potentially detect small tumors usually undetectable by abdominal ultrasound and other conventional tumor markers [26]. Their initial study focused on evaluating the clinical value of *hTERT* mRNA assay in serum of patients with HCC. Cancer, chronic hepatitis, and cirrhotic patient sera, as well as sera from healthy control individuals, were assayed. Nearly 90 % of cancer, 70 % of cirrhotic, and 41.7 % of chronic hepatitis patient samples had detectable *hTERT* message in circulation, compared to none in healthy controls. Multivariate analysis showed an independent association of circulating *hTERT* transcripts with AFP positivity, tumor size, and degree of differentiation [26]. In a follow-up study, this group developed a quantitative method to measure *hTERT* mRNA levels in serum. Serum *hTERT* mRNA was much higher in HCC patient samples than those from people with chronic liver diseases. For detection of HCC, serum *hTERT* mRNA levels performed at a sensitivity of 88.2 % and a specificity of 70 %, which is much superior to other tumor markers such as AFP levels, *AFP* mRNA, and des-gamma carboxyprothrombin (DCP). In multivariate analysis, *hTERT* mRNA levels independently correlated with tumor size and degree of differentiation as noted in previous studies [27]. In yet another study by this group, a large-scale multicenter validation study of serum *hTERT* mRNA for HCC detection and related clinical value was undertaken. Serum *hTERT* mRNA was detected in samples from HCC patients and achieved a sensitivity of 90 % and a specificity of 85.4 %, which again was much superior to other HCC biomarkers (AFP, AFP-L3, and DCP). Serum *hTERT* transcript detection was also superior to the conventional markers used for the detection of small curable tumors. In a multivariate analysis, *hTERT* mRNA had significant independent correlation with tumor size, and differentiation status, confirming previous findings by these investigators [28].

Transcripts of *TGFβ1* and protein levels in peripheral blood were assayed in relation to HBV status and their potential utility for HCC diagnosis [29]. Both tissue and blood samples were available for study. *TGFβ1* expression was present in 83.3 % of HCC and 43.3 % of adjacent noncancerous tissues (evidence of field cancerization). This finding was also more associated with HBV DNA-positive (94.7 %) than HBV DNA-negative (63.6 %) status. There was also a relationship of *TGFβ1* expression with the degree of differentiation and HBV replication. The expression of *TGFβ1* (both mRNA and protein) in circulation was significantly higher in patients with HCC than those with benign liver diseases. At levels >1.2 ug/L cutoff, *TGFβ1* levels detected HCC at a sensitivity of 89.5 % and specificity of 94 %. Together with AFP, sensitivity increased to 97.4 %.

8.4.5 Circulating HCC Noncoding RNA Biomarkers

MicroRNAs play important roles in liver carcinogenesis, as oncomirs, tumor suppressormirs, and miRNAs that coordinate or modulate viral risk factor events. Oncomirs are upregulated in HCC, and these serve to target and degrade established tumor suppressor genes. Many miRNAs are upregulated in HCC, but miR-21 and miR-221/222 homologues have such critical roles in HCC. MiR-21 is upregulated in many cancers including HCC. This miRNA targets programmed cell death 4 (*PDCD4*) and induces EMT via AKT and ERK pathway signaling. The highly homologous miR-221 and miR-222 are upregulated in HCC and also target PTEN/AKT signaling of the PI3K pathway to enhance tumor progression. Noteworthy, numerous oncogenic miRNAs of HCC are uncovered with target genes that include *MYC*, *BCL2*, *BIM*, *E2F*, *CCND1*, *CDKN1B*, *CDKN1C*, *RHOA*, and *TLR*. Several miRNAs are also downregulated in HCC, and these have tumor suppressor functions. MiR-99a-1, miR-199a-2, and miR-199b family target HIF1α, mTOR, and MET to reduce liver cancer cell proliferation, resistance to hypoxia, and enhanced invasive capacity. Loss of miR-122 in model organisms leads to liver inflammation, fibrosis, and development of HCC-like tumors. MiR-122 targets several genes including *CCNG1* with subsequent alterations in p53 tumor suppressor functions.

The clinical relevance of circulating miRNAs in HBV and cirrhosis-associated HCC has been addressed by a number of studies. MiR-122 is putatively a liver-specific miRNA. Circulating levels are increased in patients with HCC, chronic hepatitis, and cirrhosis [30]. Serum miR-21, miR-122, and miR-223 have been identified as biomarkers of HCC with individual performances that achieved an AUROCC of 0.81 for miR-21, 0.79 for miR-122, and 0.86 for miR-223. They could also detect chronic HBV infection with AUROCC of 0.91 for miR-21, 0.93 for miR-122, and 0.88 for miR-223 [30]. Qi et al. provided corroborative evidence for elevated miR-122 in differentiating HBV-associated HCC from controls at an AUROCC of 0.869 [31]. Serum miR-122 levels differentiated HBV-mediated HCC from HBV-infected liver and healthy controls at 81.6 % sensitivity and 83.3 % specificity, and the levels dropped after surgical tumor removal. The elevated serum miR-122 was significantly associated with shorter OS and was an independent predictor of poor prognosis in HCC patients (HR 1.903) [32]. In addition to miR-122, Gui et al. identified miR-885-5p as a biomarker of liver disease as well [33]. Serum miR-885-5p, however, differentiates HCC from other liver pathologies (chronic hepatitis B infection, cirrhosis) at a sensitivity of 90.53 % and specificity of 79.17 % (AUROCC 0.904).

Several other altered miRNAs are profiled in circulation of patients with HCC and demonstrated to have potential clinical utility. The ratio of miR-92a/miR-638 in plasma is much lower in HCC patients than in controls, and this ratio increased after surgical tumor removal [34]. Deep sequencing by Li et al. of sera uncovered miR-25, miR-375, and let-7f as diagnostic biomarkers of HBV-related HCC, with diagnostic sensitivity of 99.1 %, specificity of 97.9 %, and AUROCC of 0.9967 [35]. Indeed, miR-375 as a single biomarker achieved a sensitivity of 100 % at a

specificity of 96 %. A combination of miR-10a, miR-223, and miR-375 could detect HBV infection at sensitivity of 99.3 % and specificity of 98.8 %. Of interest, further discrimination of HBV infection from HBV-associated HCC was possible using a panel of serum miR-10a and miR-125 at a sensitivity and specificity of 98.5 % each and AUROCC of 0.992. Serum miR-16 and miR-199a could discriminate HCC patients from people with chronic liver disease. While both were superior to conventional serum markers, miR-16 when in a panel with conventional serum markers had the optimal sensitivity of 92.4 % at a specificity of 78.5 %. More importantly, 69 % of cases missed by conventional serum markers were detectable using serum miR-16 [36]. Zhou et al. used microarray to identify seven miRNAs (miR-21, miR-26a, miR27a, miR-122, miR-192, miR-223, and miR-801) altered in plasma from HCC patients. This miRNA panel achieved a diagnostic AUROCC of 0.864 and 0.888, respectively, in the training and validation samples, and this performance was in disregard of disease status. Additionally, the panel could differentiate HBV-mediated cancer from healthy controls at a sensitivity of 83.2 %, specificity of 93.9 %, and AUROCC of 0.941, as well as from HBV infections at sensitivity of 76.4 %, specificity of 79.1 %, and AUROCC of 0.842. It even achieved an AUROCC of 0.884 for differentiating cancer from cirrhotic patients. The promising roles of miR21, miR-122, and miR-223 in HCC require in-depth investigation.

8.4.6 Circulating HCC Protein Biomarkers

8.4.6.1 Serum Alpha-Fetoprotein as HCC Biomarker

Alpha-fetoprotein (AFP) is a glycoprotein of 70 kDa. Specific fetal tissues, including liver cells of the vitelline sac and fetal intestinal cells, normally synthesize it during early (first trimester) fetal development and later by hepatocytes and endodermal cells of the yolk sac. It is normally downregulated after birth. But expression of AFP is increased in pathologic states such as liver regeneration, HCC, or embryonic carcinomas. Functionally, AFP is a carrier molecule like albumin in blood, which is why it is sometimes called alpha-fetoglobulin.

The measurement of AFP is the "gold standard" of circulating HCC biomarkers. However, it has its own deficiencies as an ideal clinical biomarker. In normal individuals, the serum levels rarely exceed 20 ng/ml; however, inflammatory liver diseases and cirrhosis lead to elevated levels, which sacrifices specificity for HCC. Levels over 400 ng/ml, however, can be diagnostic for HCC, but this as well hampers sensitivity. Because of these factors, several cutoff values are used in various studies. The circulating levels of AFP depend on several factors including tumor size. Thus, the sensitivity is ~52 % for tumors $>$ 3 cm, but this drops to 25 % for small tumors ($<$3 cm). Many (~80 %) small tumors are not associated with elevated levels in the circulation, limiting its use as an early detection biomarker.

Thus, AFP is not a routine screening biomarker, even in endemic geographic regions. It is recommended however for:

- Screening of high-risk individuals (e.g., people with HBV and HCV infections and cirrhosis) in conjunction with abdominal ultrasound.
- Monitoring tumor behavior following treatment. In patients with AFP-secreting HCC, declining levels to normal after therapy indicates response and vice versa. Treatment efficacy requires that AFP levels decline by 10 ng/ml within 30 days of treatment. To be useful in this setting, levels must be elevated prior to commencing treatment.
- AFP complements tumor staging. AFP is used in the "Cancer of the Liver Italian Program" (CLIP) staging system. This staging system enables the assessment of liver function and tumor features in relation to prognosis and therapy response prediction.

8.4.6.2 Serum AFP-L3 as HCC Biomarker

AFP glycoproteins have different binding affinities to lectins, such as lens culinary agglutinin (LCA). There are three LCA fractions of AFPs with different binding affinities:

- *Lens culinaris* agglutinin fraction 1 (AFP-L1) or LCA nonreactive has no affinity for LCA. This is the primary isoform in sera of people with chronic hepatitis and liver cirrhosis.
- *Lens culinaris* agglutinin fraction 2 (AFP-L2) has intermediate binding affinity to LCA. It is detectable in sera from pregnant women and people with yolk sac tumors.
- *Lens culinaris* agglutinin fraction 3 (AFP-L3) glycoform, also called LCA-reactive fraction 3 of AFP, has the highest affinity to LCA. This isoform comprises 1–6 fructoses attached to the reducing terminus of *N*-acetylglucosamine. AFP-L3 is the specific isoform of AFP to HCC.

At cutoff levels of >10 % of total AFP, AFP-L3 has a sensitivity of 51 % and a specificity of 95 % for detection of HCC. It is normally used to complement diagnosis of HCC in individuals with elevated total AFP. Elevated serum levels mirror tumor progression. Levels increase progressively in patients with moderately, to those with poorly differentiated tumors. Similar to AFP, sensitivity depends on tumor size, being 35–45 % for tumors $<$ 2 cm and 80–90 % for larger tumors $>$ 5 cm. Thus, AFP-L3 is also used clinically to assess tumor invasiveness and metastasis, histologic grade, prognostic prediction, and monitoring after treatment. AFP-L3 positivity is associated with poorly differentiated tumors, with early vascular invasion, and with intrahepatic spread. Additionally, expression is associated with aggressive and invasive phenotypes evidenced by their correlation with increased nuclear Ki67 and decreased α-catenin expressions. Following treatment,

patients positive for AFP-L3 have shorter survival time than those with undetected levels.

8.4.6.3 Serum Des-Carboxyprothrombin as HCC Biomarker

Also known as prothrombin induced by vitamin K absence II (PIVKA-II), des-carboxyprothrombin (DCP) is an abnormal prothrombin produced by hepatocytes as a result of vitamin K deficiency. It is undetectable in sera from healthy individuals, but levels are elevated in patients with HCC. Prothrombin is produced as a precursor protein with ten glutamic acid residues at its N-terminal. These amino acid residues undergo vitamin K-dependent carboxylation to gamma-carboxyglutamic acid, to produce the mature normal prothrombin. An elevated level of DCP in sera of patients with HCC is apparently a result of possibly many factors that reduce vitamin K levels. These include:

- Vitamin K insufficiency secondary to abnormal intake or intracellular transport mechanisms.
- Selective defects in gamma-carboxylase enzyme.
- Epithelial-to-mesenchymal transition as the cancer cells undergo epithelial-to-fibroblastoid conversion required for vascular invasion and intrahepatic spread. This process is associated with cytoskeletal changes that impair vitamin K uptake.

Serum DCP as HCC Diagnosis Biomarker

Des-carboxyprothrombin has similar diagnostic utility as AFP/AFP-L3. Because this tumor marker has such strong association with hepatoma cells, enhanced specificity as in AFP-L3 is expected, but sensitivity will depend on levels produced, which may mirror tumor size and invasive behavior, indicating late diagnosis. Thus, sensitivity is between 48 and 62 % and specificity is 81–98 % for the detection of HCC. Vitamin K supplementation decreases serum levels and can mask diagnosis, if unwary of this. Regarding diagnostic utility, data reported from Europe do not support its value. However, studies from North America and Asia indicate DCP is more accurate than AFP in HCC detection. Confounding factors in such geographic differences may be the various predominant etiologic agents and possible racial differences. In general, levels of DCP are elevated in just ~20 % of small tumors. The sensitivity of DCP also mirrors tumor size; for tumors < 3 cm, sensitivity is 74 % but increases to 83 % for tumors 3–5 cm and up to 96 % for larger tumors > 5 cm.

Des-carboxyprothrombin has also been used in a panel with AFP for the possible early detection HCC. The serum levels of these two biomarkers do not correlate, hence the combination of the two in a panel can increase the sensitivity of DCP up to ~80 % for large tumors (>3 cm) and ~70 % for small tumors (2–3 cm). Reasons for their complementary use are that DCP is more specific than AFP because the serum levels of DCP are less likely to increase by conditions such as chronic liver disease (increasing specificity). The levels of DCP are much superior at

discriminating HCC from cirrhosis at a sensitivity of 86 % and specificity of 93 %. But sensitivity of DCP is lower than AFP for small tumors, thus AFP provides that needed sensitivity. However, for larger tumors, DCP is more sensitive [37]. In a study where serum measurements were taken 12 months prior to and at diagnosis, DCP at a cutoff value of 40mAU/ml had a sensitivity of 43 % and a specificity of 94 % for HCC at 12 months prior and a sensitivity and specificity of 74 % and 86 %, respectively, at diagnosis. At a cutoff value of 20 ng/ml, AFP had a sensitivity and specificity of 47 % and 75 % (12 month prior) and 61 % and 81 % (at diagnosis). When used as a panel, the AUROCC was 0.92 at diagnosis compared to 0.79 for AFP alone, and 0.82 for DCP alone.

Serum DCP as HCC Prognostic Biomarker

Relapse and survival outcomes are primary concerns following HCC diagnosis and treatment. The ineffective screening procedures for early detection result in late disease detection, which is often associated with less than desired outcomes. Elevated DCP levels in circulation correlate with tumor size, vascular invasion, and intrahepatic metastasis, all of which are dismal prognostic indicators of survival. DCP is an independent prognostic factor of survival and predictor of recurrences after surgery, ablation treatment, liver transplantation, and transarterial chemoembolization treatments.

While many prognostic studies focused on larger tumors (because of lack of DCP sensitivity in small tumors), Hakamada et al. examined preoperative DCP levels in patients who underwent hepatic resection with defined criteria of small tumors ($\leq$5 cm or no more than three tumor nodules $\leq$3 cm) [38]. Multivariate analysis revealed vascular invasion and serum DCP (>400 mAU/ml) as independent prognostic factors. Of the 142 patients in this study, 85 met the criteria for small tumors, and among this group, DCP levels were the significant predictor of recurrence-free and overall survival. But for the remaining patients (with defined large tumors), vascular invasion was an independent predictive factor for recurrence-free and overall survival. In many (75 %) of those with small tumors but high DCP levels, recurrence was intrahepatic. Indeed, the association of high preoperative DCP levels, tumor size, and histologic grade or differentiation, with microscopic vascular invasion and poor prognosis, is confirmed by numerous other studies. Currently, serum AFP, DCP, tumor size and number, degree of histologic differentiation, vascular invasion, and stage are recognized significant prognostic factors of HCC.

The role of DCP, especially preoperative elevated levels, in predicting microscopic vascular invasion and outcome in small tumors, is of outmost clinical relevance. Thus, preoperative levels > 300 mAU/ml are associated with microscopic vascular invasion. A prognostic study of AFP, AFP-L3, and DCP levels at diagnosis in patients who were treated with hepatectomy or locoregional thermal ablation (LTA) was conducted with interesting findings. In the hepatectomy group, no tumor marker was associated with survival. However, high AFP-L3 or DCP levels were associated with dismal survival among those who received LTA. Similarly, DCP levels were best predictors of survival in patients who received

radio-frequency ablation therapy. Survival was significantly lower in patients with DCP $\geq$ 100mAU/ml compared to those with lower levels. The use of DCP levels to predict recurrence in HCC patients who received living donor liver transplant (LDLT) has also been addressed. Tumor size $<$ 5 cm and DCP levels $<$ 300 mAU/ml are favorable independent factors of recurrence of HCC after LDLT. This has implications for patient selection for this intervention. The Kyoto criteria for candidate selection require DCP levels $\leq$ 400 mAU/ml, $\leq$10 nodules, and tumor size $\leq$ 5 cm.

Serum DCP as HCC Staging Biomarker

DCP levels also correlate with tumor stage by TNM, CLIP, and Japan Integrated Staging (JIS) systems. The JIS score fails to predict or estimate malignant grade of HCC. Kitaki's group attempted to improve this scoring system by incorporating the three biomarkers, namely, DCP, AFP, and AFP-L3 in the JIS system, which was referred to as the biomarker combined JIS (bm-JIS) [39, 40]. In 1924 HCC patients, the bm-JIS score had a better predictive ability than the conventional JIS score. Bm-JIS is a much better prognostic predictor. In patients who undergo hepatic resection, prognosis is better predicted by the SLiDe (SLiDe is for *s*tage, S; *li*ver damage, Li; and *De*sCP levels, De) staging or predictive score system, which incorporates DCP levels.

8.4.6.4 Serum Glypican 3 as HCC Biomarker

Glypican 3 (GPC-3) is a member of the heparin sulfate proteoglycan family of the extracellular matrix. It is encoded as a 70 kDa precursor protein that is processed by furin into an N-terminal 40 kDa and a C-terminal 30 kDa protein connected by two heparin sulfate glycans. It binds to growth factor receptors and controls cellular proliferation and growth during embryonic development. Mutations of this gene cause Simpson–Golabi–Behmel syndrome. Of interest, GPC-3 is overexpressed in HCC and has a role in cancer through induction of FGF, IGF, and WNT signaling pathways and also interacts with MMPs. Its role in hepatocarcinogenesis is yet to be fully elucidated.

GPC-3 is a potential HCC diagnostic biomarker. Hepatoma cells, but not normal hepatocytes and cells of benign liver diseases including cirrhosis, express GPC-3. This suggests its potential specific use in detection of HCC. Serum levels were $>$300 ng/L in 50 % of early stage HCC patients who had AFP levels $<$ 100ug/L. GPC-3-positive cells were found in 90 % of patients with serum AFP $<$ 400ug/L, and serum levels were elevated in early stage HCC patients with serum AFP levels $<$ 400 ug/L [41]. Qiao et al. measured serum GPC-3, AFP, and human cervical cancer oncogene (HCCR) as diagnostic biomarkers of HCC [42]. Using a diagnostic cutoff value of 26.8 ng/ml, 58.8 mAU/ml, and 199.3 ng/ml for GPC-3, HCCR, and AFP, respectively, the sensitivities were 51.5 % (GPC-3), 22.8 % (HCCR), and 36.6 % (AFP) at specificities of between 90.9 and 98.5 %. The three biomarkers as a panel achieved a sensitivity of 80.2 %. As a single biomarker,

however, the diagnostic utility of GPC-3 has been dismal compared to AFP alone [43].

8.4.6.5 Serum Osteopontin as HCC Biomarker

Osteopontin (OPN) is an extracellular matrix secreted glycophosphoprotein that interacts with integrin alpha V subunits and CD44 family of receptors. Initially it was named SPP1, for secreted phosphoprotein 1, because it was the major phosphoprotein secreted by cultured cells. Several cell types, including osteoblasts, smooth muscle cells, and epithelial cells, cells of the immune system, and tumor cells, produce this molecule. Osteopontin belongs to the small integrin-binding ligand N-linked glycoprotein (SIBLING) family and has an arginine–glycine–aspartate (RGD)-containing acidic group. The interactions of OPN with integrin and CD44 family of receptors mediate cell signaling that controls inflammatory processes, as well as tumor progression and metastasis. It is involved in hepatic inflammatory conditions such as hepatitis and has potential utility as a biomarker of HCC.

Osteopontin is secreted into plasma, and its noninvasive diagnostic and prognostic use has been explored. Plasma OPN levels are significantly higher in HCC patients than healthy controls and people with benign liver diseases. Plasma levels are suggested to outperform AFP in HCC detection, especially in viral-mediated HCC. Used as a panel with AFP (cutoff of 20 ng/ml) and OPN (cutoff 156 ng/ml), HCC is detectable at a sensitivity of 95 % and specificity of 96 %. High levels have prognostic application as well, because they are associated with decreased liver function, advanced tumor stage, vascular invasion, and intrahepatic metastasis. Such elevated levels predict poor overall and disease-free survival, and levels above 200 ng/ml are associated with postoperative recurrence.

8.4.6.6 Serum Dickkopf-1 as HCC Biomarker

Diskkopf-related protein 1 (DKK1) is a canonical WNT/b-catenin pathway inhibitor. As a secreted protein, circulating levels are elevated in people with osteolytic bone lesions such as in multiple myeloma. DKK1, however, appears to have a complex role in cancer. Its role in HCC has been examined. In a large retrospective cross-sectional survey. Shen et al. demonstrated the utility of serum measurements of DKK for the early detection of HCC [44]. After establishing a cutoff for HCC detection using samples from 424 patients and 407 control subjects, the usefulness of DKK in HCC detection was determined. Serum levels of DKK were significantly more elevated in HCC patients than control individuals. The high circulating levels appear more useful for detection of AFP-negative HCC. In combination with AFP, however, the detection rate of HCC was enhanced [44].

8.4.6.7 Serum Midkine as HCC Biomarker

Midkine (neurite growth-promoting factor 2) (MDK) belongs to a small family of secreted growth factors. It plays a role in cell growth, invasion, and angiogenesis during cancer progression. The serum levels of MDK have been evaluated in HCC. The elevated levels of this biomarker appear superior to the gold standard, AFP, for HCC detection [45]. Serum levels were elevated in a large number of patients with HCC, and these levels declined following curative intent surgery but remained or became elevated in patients with recurrent diseases. With regard to HCC detection, MDK is more sensitive (86.9 %) than AFP (51.9 %), but they both demonstrate similar specificities (83.9 % for MDK vs. 86.3 % for AFP). MDK appears more sensitive (80 %) at early HCC detection than AFP (40 %) and even adapt to early cancer detection in patients with AFP-negative outcomes at a sensitivity of 89.2 %.

8.4.7 Circulating HCC Cells

Pioneering studies of circulating HCC cells (CHCCCs) have used molecular methods targeting AFP mRNA. Other molecular markers (e.g., MAGE) and the CELLSEARCH® system have been successfully applied for CHCCC enrichment and characterization as well.

CHCCCs are associated with various clinicopathologic parameters. CHCCCs were detected in 52 % patients with primary liver cancer, 15 % cirrhotics, and in 12 % of patients with chronic hepatitis but not in any of the healthy controls [46]. The presence of CHCCCs was associated with tumor size and serum AFP levels. A subsequent study focused on the prognostic relevance of CHCCCs. The probability of extrahepatic metastasis was higher in patients with positive AFP mRNA. Following transarterial embolization, persistent CHCCC detection was associated with poor overall and metastasis-free survival [47]. Louha et al. examined the spontaneous and treatment-related (i.e., chemoembolization or alcoholization) release of CHCCCs [48]. While all controls were negative for AFP mRNA, 33.3 % of patients with primary liver cancer tested positive. This spontaneous liver cancer cell release was associated with increased serum AFP levels, extrahepatic spread, tumor size, portal thrombosis, and intravascular tumor emboli. Of the patients who received locoregional therapy, half were positive for CHCCCs 1 and 24 h after treatment. Liu et al. provided corroborative evidence to previous workers [49]. In their series, *AFP* mRNA was detected at a rate of 53.8 %, and this was associated with serum AFP, tumor size, TNM stage, intra- and extrahepatic metastasis, and portal vein thrombosis.

Other findings of CHCCCs include their strong association with disease recurrence. Wong et al. demonstrated the release of cells (both normal and malignant) into circulation at hepatectomy [50]. But the persistence or increase in CHCCs after surgery was predictive of poor outcome, due to intrahepatic and extrahepatic

metastasis and recurrence. A later study with increased number of patients arrived at a similar conclusion as in the pilot study of 1999 [51]. The release of cells at surgery may be responsible for disease recurrence/metastasis after surgery. Additionally, *AFP* mRNA-positive CHCCCs were predictive of metastasis and recurrence in 56 % of cases during a 4-year follow-up. AFP mRNA was positive in 25.6 % of HCC patient samples, and this was associated with multiple intrahepatic nodules. High AFP transcripts predicted recurrence [52]. Schmilovitz-weiss et al. performed a small sample analysis, but pre- and post-treatment AFP mRNA levels predicted local and distant recurrences [53]. Zhang et al. attempted to increase the specificity of CHCCC detection by including other tumor markers [54]. First, three tumor markers, *MAGE1*, *MAGE3*, and *AFP* mRNA, were targeted and detected CHCCCs at a rate of 14 %, 20.1 %, and 33.7 %, respectively. In combination, at least one marker was positive in 52.3 % of cancer patients. Tumor marker detection correlated with TNM stage, extrahepatic metastasis, and portal vein tumor thrombosis [54]. In a follow-up study correlating *MAGE1* and/or *AFP* mRNA detection in blood to clinical outcome, persistent positivity of *MAGE1*/*AFP* transcripts after surgery (as well as those who converted to positive status after surgery) was significantly associated with recurrence or metastasis (88.1 % compared with 3.6 % of those negative for markers, $p < 0.001$) [55].

The prognostic potential of CHCCCs has also been examined. *AFP* mRNA was detected in 27.2 % and 23.4 % of pre- and post-operative patients with HCC. However, only postoperative presence of CHCCCs significantly predicted recurrence-free survival in multivariate analysis (HR 3.13, $p = 0.002$) [56]. In HCC patients without evidence of extrahepatic metastasis, *AFP* mRNA was detected in 40 % of patients, and this was associated with tumor size, vascular invasion, and moderate- to- poor differentiation. *AFP* mRNA was also significantly associated with poor overall survival. In this study, which included patients with colon cancer, pancreatic cancer, and cirrhosis, *AFP* transcripts were detected in 36 % of patients (thus not a specific marker of HCC as suggested by others) [57]. Morimoto et al. examined CHCCCs/DTCs before and after liver resection and correlated them with clinicopathologic features and prognosis [58]. CHCCCs were detected at a rate of 23.7 %, while DTCs were in 48 % of patients. However, only CHCCCs significantly correlated with extrahepatic metastasis and shorter disease-free survival after surgery. In another series, DTCs were positive in 27.9 % of primary live cancer patients who underwent curative intent hepatectomy. On multivariate analysis, DTCs significantly predicted patient survival or disease-free survival. Positive CHCCCs also were predictive of DFS [59].

Despite the demonstrated potential of CHCCCs, others have negative findings on their clinical relevance. In one study, *AFP* mRNA in HCC patients who received various therapeutic maneuvers or not was not clinically relevant. While detection of CHCCCs was significantly higher in cancer patients (28 %) than controls (3 %), their presence did not predict disease recurrence [60]. In another study, CHCCCs were detected at the same frequency before and after transarterial chemoembolism. While extrahepatic metastasis was significantly associated with positive *AFP* mRNA, it did not affect metastasis-free survival or increase the risk of extrahepatic

recurrence [61]. Also the use of central venous blood may not be suitable for CHCCC detection for clinical applications. Central venous blood tested positive for *AFP* mRNA in both HCC patients and healthy volunteers, questioning its value for CHCCC evaluation [62]. However, bone marrow samples from patients were positive for CHCCCs in 16.7 % of HCC patients. These discrepant findings may partly be attributable to study design including attributes of the patients and healthy controls.

Other methodologies and molecular targets have been explored for CHCCC studies. Vona et al. first questioned the ability to detect CHCCCs, as well as the clinical relevance of these cells, using cytomorphometry followed by isolation by size [63]. The study population included patients with nonmetastatic primary liver cancer, healthy controls and patients with chronic hepatitis, and cirrhosis. CHCCCs and microemboli were detected in 52.3 % of cancer patients and none in the controls. CHCCCs were associated with tumor spread and portal vessel tumor thrombus, and CHCCCs and tumor microemboli were associated with shorter survival [63]. Schulze et al. used the established CELLSEARCH® technology to characterize CHCCCs for prognostic relevance [64]. CHCCCs were recovered in 30.5 % of patients, and this was associated with shorter overall survival time (460 vs. 746 days). CHCCC detection correlated with vascular tumor invasion and advanced Barcelona Clinic Liver Cancer stage C disease [64]. The prognostic utility and possible stem cell presence in the population of EpCAM-positive epithelial cells in circulation of HCC patients receiving curative intent surgery were investigated [65]. CHCCCs were detected in 66.67 % of this cohort, with detected cell numbers ranging from 1 to 34/7.5 mls of blood. Preoperative CHCCCs $\geq$ 2 were significantly associated with tumor recurrence in contrast to those with <2 CHCCCs/7.5 mls of blood. Significant reductions in CHCCC detection rates (66.67–28.15 %) and numbers (2.60 $\pm$ 0.43 to 1.00 $\pm$ 0.36) were observed 1 month after surgery. Persistent CHCCC counts $\geq$ 2 were a strong indicator of recurrence (81.7 % of cases vs. 15.5 % of those with <2 CHCCCs). The possible inclusion of stem cells and biologic features of CHCCCs were the finding that some CHCCCs were CD133 and ABCG2 positive, demonstrated EMT, activated WNT signaling, had increased tumorigenicity with antiapoptotic characteristics [65]. Xu et al. developed a sensitive method for CHCCC detection, which enabled a recovery of circulating cells in 81 % of their patients (average 19 $\pm$ 24 cells/5 ml of blood). CHCCC positivity and number significantly correlated with tumor size, differentiation status, TNM stage, and portal vein tumor thrombosis. The isolated CHCCCs were characterized for *TP53* mutations and deletions and *HER2* amplification [66].

Meta-analysis of CHCCCs for diagnostic and prognostic use included just five publications (four PhD theses and one published data) involving 535 liver cancer patients [67]. The conclusion was that CHCCC detection was significantly associated with tumor size (>5 cm), late clinical stage (III/IV), and presence of metastasis. Another systematic review observed the small sample sizes and heterogeneous nature of many studies [68]. The potential value of CHCCCs in patient management therefor waits further evaluation.

8.5 Summary

- The majority of HCCs occurs in Sub-Saharan Africa and Southeast Asia, where established risk factors such as aflatoxins, HBV infections, and chronic hepatitis are prevalent.
- The 5-year survival is optimal at ~70 % with early cancer detection.
- In the resource-rich parts of the world, people at elevated risk are screened for using US, CT scans, and MRI modalities.
- In the resource-poor regions of the world, screening relies on serum measurements of AFP and DCP levels. While useful, these biomarkers lack the desired accuracy as screening biomarkers.
- The molecular pathology of HCC includes chromosomal, epigenetic, and genetic alterations in members of the RAS-MAPK, PI3K, WNT/β-catenin, and cell cycle pathways.
- The altered molecules serve as biomarkers that are detectable in circulation of cancer patients.
- Apart from the traditional serum proteins, serum levels of glypican 3, OPN, DKK1, and MDK demonstrate some clinical potential.
- Advanced stage HCC is associated with CHCCCs, which have been targeted primarily using molecular approaches. Their prognostic value needs validation studies.

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Chapter 9
Pancreatic Cancer Biomarkers in Circulation

Key Topics

- Challenges with pancreatic cancer (PanCa) screening
- Screening for PanCa
- Molecular pathology of PanCa
- Circulating PanCa miRNA biomarkers
- Circulating PanCa proteomic and metabolomic biomarkers
- Circulating PanCa cells

Key Points

- Pancreatic cancer (PanCa) has a high mortality rate primarily due to late diagnosis. The pancreas cannot easily be palpated and yet there are no validated noninvasive screening biomarkers, resulting in late stage presentations.
- The established molecular pathologic changes in PanCa are detectable in ctDNA, and some including *KRAS* mutations are adjuncts to clinical decision-making in patient management.
- Several circulating biomarkers including miRNAs, traditional serum proteins, and proteomic profiles as well as circulating PanCa cells are potential actionable clinical biomarkers.

9.1 Introduction

Pancreatic cancer (PanCa) is the tenth most diagnosed cancer in the United States and yet is the fourth cause of cancer-related fatalities. The 2012 global estimated incidence for PanCa was 337,760 with an associated mortality of 330,372. In the

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United States, similar dismal outcomes are consistently being reported with an estimated 53,070 new cases and 41,780 deaths in 2016. The global age-standardized incidence and mortality are 3.9/100,000 and 3.7/100,000, respectively. Thus, the incidence is similar to the mortality rate, and this has remained so for several decades. Indeed, mortalities from many cancers such as breast, prostate, and lung cancers are declining over the past decades, but those from PanCa have remained the same or are increasing, especially in the elderly (>70 years). The reason for this poor outcome is the absence of screening programs for the general population at low to medium risk, and thus many tumors are being diagnosed at an advanced stage, which are not amenable to surgery, and yet there is no effective alternative therapy. While there is no consensus on who, when, and how often screening should be done, several centers use their own discretion, with many urged to only screen those with a relative risk score of >10 (out of 14.3). Because many people at risk are excluded from screening, over 80 % of cases are detected at an advanced unresectable state, often associated with distant metastasis. In view of such late presentations, the majority (>74 %) of patients die within 12 months of initial diagnosis, and up to 94 % die within 5 years. However, the 5-year survival rate can be as high as 75 % when small tumors (<1 cm) are detected. Indeed, a screening program of high-risk individuals can potentially lead to early detection of all patients, with associated excellent cure rates. Thus, early detection of organ-confined small tumors should improve the outlook for PanCa patients. Additionally, PanCa is a latent disease that develops over several years after acquiring the initial genetic alterations. Modeling of sequencing data from primary and metastatic PanCa suggests it takes ~17 years for a cell to progress from initiation to metastasis. There is thus a wide window of opportunity for early detection, using molecular biomarkers in circulation. The need for noninvasive early detection biomarkers is thus eminent, and there are potential targets worthy of validation for product development.

9.2 Challenges with PanCa Screening

In spite of the rise in incidence and mortality, the screening for early detection of curable PanCa is far from optimum. The simplest reason is the lack of validated cost-effective serum biomarkers for population-based screening. Additionally, sensitive tissue-specific biomarkers detectable in pancreatic juice where they are enriched for cannot be applied to the entire population at risk (i.e., people over 30 years of age), because obtaining such fluid is invasive and expensive, requiring endoscopic retrograde cholangiopancreatography and sampling. A gastroduodenal capsule is an alternative minimally invasive means of obtaining pancreatic juice but will need to overcome some important hurdles including:

- The sampling device will need validation.
- A possible pharmacologic induction of pancreatic juice secretion.
- This may require identification/validation of pancreatic cancer-specific biomarkers distinct from those of the hepatobiliary system.

Imaging modalities, especially molecular imaging, will offer a better noninvasive approach. However, molecular imaging is cost prohibitive for mass screening, and there are currently only experimental data with no biomarkers or probes developed for such utility. Thus, numerous efforts at biomarker identification for screening have focused on blood components. While this probably offers the best approach, there are inherent challenges with biomarker specificity.

9.3 Screening Recommendations for PanCa

Several risk factors are identified for the development of PanCa. Some, such as age, gender, race, smoking, alcohol, and obesity, have overlap risks with several other cancers. Pancreatitis and diabetes are associated with increased risk for PanCa. Allergies are putatively associated with 30–45 % reduced risk. Moreover, genetic polymorphisms especially non-O blood group type alleles increase the risk for PanCa. Also implicated is *H. pylori* infection. However, the highest risk is among people with familial syndromes (Table 9.1). But, only 3–16 % of all PanCas are

Table 9.1 Hereditary syndromes associated with risk of PanCa

Hereditary syndrome	Genes involved	Relative risk	Extra-pancreatic involvement
Familial pancreatic syndrome	Unknown	4.6–32	–
Familial adenomatous polyposis	*APC*	4	Colorectum, small intestines, stomach
Familial atypical multiple mole melanoma	*CDKN2A*	9–22	Skin (melanoma)
Hereditary breast and ovarian syndrome	*BRAC1*, *BRAC2*, *FANCC*, *FANCG*, *PALB2*	3.5–10	Breast, ovary, prostate
Hereditary pancreatitis	*PRSS1/TRY1*, *SPINK1*	~50	–
Hereditary nonpolyposis colorectal carcinoma syndrome	*MLH1*, *MSH2*	8.6	Colorectum, small intestines, endometrium
Peutz–Jeghers syndrome	*ST11/LKB1*	~132	Small intestines, colorectum, esophagus, stomach, bile duct, lungs, breast, ovary, uterus
Li–Fraumeni syndrome	*TP53*		Several

familial or syndromic with well-established defined gene alterations. The majority are therefore sporadic cases.

Screening recommendation from the fourth International Symposium on Inherited Diseases of the Pancreas is for healthy people with at least three affected first-degree relatives or *BRCA2* mutation carriers with at least one family member diagnosed with PanCa. Screening should begin at age 50 or at an age 10 years earlier than the age at which the youngest family member was diagnosed. Also consensus practice recommendation suggests an increased risk of tenfold should guide screening (but this tenfold cutoff implies several people with elevated risk are excluded from screening). Screening frequency is not established, and screening algorithms or procedures are not standardized. Currently, screening involves a multidisciplinary team of experts using various imaging techniques.

Unlike other cancers such as breast and prostate that can be detected by palpation on physical examination, and gastrointestinal tract tumors that can easily be visualized with an instrument by an expert for possible early detection, the retroperitoneal anatomic location of the pancreas precludes easy access. In view of this, and coupled with the various advances made in imaging modalities, imaging has been the primary tool for the clinical evaluation of pancreatic masses, and screening of those with elevated risk for developing PanCa. While endoscopic ultrasound (EUS) or magnetic resonance imaging (MRI) is initially used, and if abnormal other modalities are deployed, the eventual diagnosis requires EUS-guided fine needle aspiration and histopathologic visualization of cancer cells. Various other imaging modalities including computerized tomography (CT) and positron-emission tomography (PET) scans, endoscopic retrograde cholangiopancreatography (ERCP), and magnetic resonance cholangiopancreatography (MRCP) are used by various centers for screening. Effective screening algorithm with imaging, combined with genetic testing, proves useful for early detection, not only of PanCa but neoplastic lesions outside of the pancreas such as ovarian, papillary thyroid cancer, and carcinoid tumors. The best imaging modality is EUS. This is more accurate than the other modalities at viewing deep-seated masses. When combined with sampling of the mass by fine needle aspiration, EUS offers a high sensitivity of 92 % for PanCa detection. This imaging modality is superior to CT scans and MRI for detecting lesions <1 cm and intraductal papillary mucinous neoplasm (IPMN). EUS is also better at resolving lymph node metastasis and vascular infiltration compared to CT scans.

While early detection with curative intervention strategies will obviously reduce mortalities from PanCa, screening is impractical at present for the entire asymptomatic population at risk due to cost prohibition, invasiveness of screening procedures that can be associated with complications such as pancreatitis, lack of proven sensitivities, and the generally low disease prevalence. The need for accurate noninvasive and cost-effective biomarkers for screening and triaging of asymptomatic individuals for more invasive assessment is therefore eminent. Such

biomarkers in circulation will be ideal. While many are discovered, validation, product development, and widespread clinical translation are awaited.

9.4 Molecular Pathology of PanCa

Many PanCas (~65 %) are localized to the head of the pancreas and may present early with signs of pancreatic ductal obstructions (Fig. 9.1). However, the remaining 35 % occur elsewhere in the pancreas. Putatively, the anatomic location may be associated with prognosis, with cancers at the tail being associated with increased in-hospital complications and mortality, probably due to late diagnosis of advanced diseases.

9.4.1 Familial and Sporadic PanCa

While the majority of PanCas occur without any relationship to familial segregation (sporadic PanCa, SPC), up to 10 % of all PanCas have some hereditary components (familial PanCas, FPC). The genetic landscape of SPC is well elucidated; however, FPC is often associated with genes involved with other cancer and disease syndromes (Table 9.1). Familial PanCa is considered when at least a pair of first-degree relatives (two siblings or a sibling and a parent) is diagnosed with PanCa. It has been estimated that the risk of developing PanCa is 18-fold in healthy people from

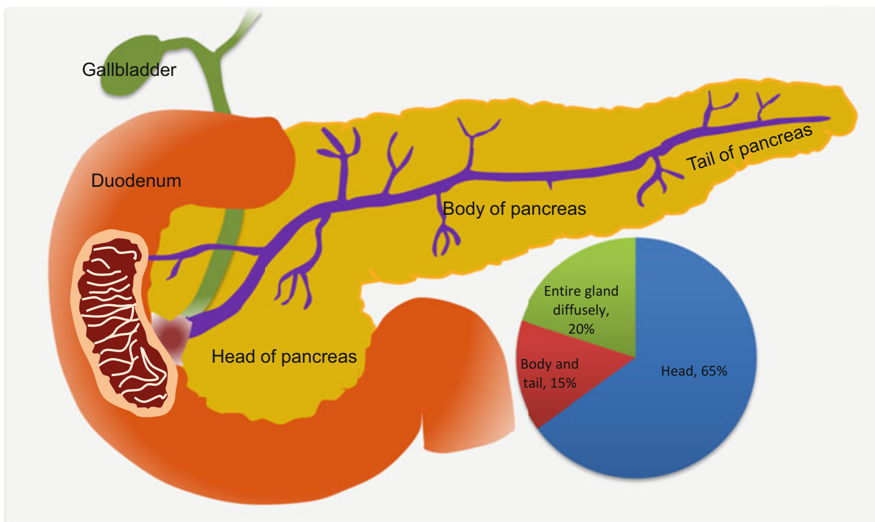

Fig. 9.1 Frequencies and anatomic locations of PanCas

these families, and this increases to 56-fold when the disease has afflicted $\geq$3 family members.

Implicated in the pathogenesis of FPC are alterations in genes involved in the associated syndromes (Table 9.1), as well as *PALLD*, *PALB2*, and *PHOX2B*. It may also appear that the genetics of FPC cases of late onset have identical molecular pathology as SPC. Norris et al. used whole exome, whole genome, and RNA sequencing together with high-density SNP arrays to study cell lines and matched germline samples from a cohort of FPC patients and revealed that the four major driver genes of SPC, *KRAS*, *TP53*, *CDKN2A/p16*, and *SMAD4/DPC4* were consistently altered in these samples as well [1].

9.4.2 Molecular Subtypes of PanCa

Work by Collisson and colleagues on gene expression analysis enabled the classification of PanCa into three distinct molecular subtypes, namely, classic, quasimesenchymal, and exocrine-like [2]. These subtypes also differ in terms of their response to treatment and clinical outcome:

- The classic subtype overexpresses high levels of adhesion-associated and epithelial genes, including *S100PBP*, *AGR2*, *CEACAM5*, *CEACAM6*, and *FXYD5*. Tumors of this subtype are more sensitive to oral EGFR tyrosine kinase inhibitors such as erlotinib.
- The quasimesenchymal tumors express high levels of mesenchyme-associated genes such as *TWIST1*, and *S100A2*. They are more sensitive to gemcitabine therapy and also have worse outcome than the classic subtype.
- The exocrine-like PDACs express high levels of genes involved with digestion such as *REG3A* (PAP), *PRSS1/2*, *SLC3A1*, and *CFTR*. It has been demonstrated that tumors of this subtype that express *CYP3A5* are resistant to TKIs [3].

9.4.3 Genetics of PanCa

There are marked geographic and racial variations in PanCa incidence, being high among African-American men in particular and also in Koreans, Czechs, Native Hawaiians, New Zealand Maoris, and Latvians. Globally, PanCa ranks 13th in incidence and 8th case mortality among cancers. However, the high incidence in the identified populations is not necessarily explained by known risk factors, making genetic factors the probable culprits.

9.4.3.1 Intragenic Point Mutations in PanCa

Almost all PanCas demonstrate intragenic mutations in genes involved in several signaling pathways. Oncogenes (*KRAS2* and *BRAF*), TSGs (*CDKN2A*, *TP53*, and *SMAD4*), and caretaker genes (*BRAC2*, *FANCC*, and *FANCG*) are all mutated at various frequencies in a variety of PanCas. *KRAS2* mutations occur in as many as 95 % of PDACs. Interestingly, cancers without *KRAS2* mutations tend to harbor *BRAF* mutations, indicating an activation of the RAS/BRAF/MEK/ERK pathway in almost all PDACs. Other MAPK pathway mutations include *STK11/LKb1* (of Peutz–Jeghers syndrome). TGFβ/activin pathway mutations involve *SMAD4/DPC4*, *TGFβRI*, *TGFβRII*, *ACVR1B*, and *ACVR2*. The Fanconi anemia pathway is involved by mutations in *BRAC2*, *FANCC*, and *FANCG*.

9.4.3.2 Important Oncogenes and Tumor Suppressor Genes in PanCa

KRAS Alterations in PanCa

The *KRAS* oncogene is mutated at a high frequency in PanCa. *KRAS* gene on chromosome 12 encodes KRAS protein, which is a member of the RAS family of GTP-binding proteins that control cellular proliferation, growth, cytoskeletal remodeling, motility, differentiation, and survival. *KRAS* is the most frequently mutated gene in PanCa, occurring in almost all (>95 %) PanCas. Mutations are early events in PDAC, being detected in PanIN1. Activating mutations in this oncogene, especially in codon 12, leads to constitutive KRAS activity, which includes activation of RAF family of serine threonine kinases and eventual downstream BRAF-MAPK and PI3K/AKT signaling. The final output of such a cell is acquisition of uncontrolled division and growth, evasion of death signals, and eventual development of invasive phenotype. The few (~5 %) of PanCas without *KRAS* mutations harbor $BRAF^{V600E}$ point mutations and are often microsatellite unstable.

CDKN2A Alterations in PanCa

The *CDKN2A* tumor suppressor is also inactivated in many (~95 %) PDACs. Located on chromosome 9, one product of this gene is the p16 protein that inhibits cyclin-dependent kinase 4- and 6-mediated phosphorylation of retinoblastoma (RB). This prevents entry into the S phase of the cell cycle (G1-S check point). Thus *CDKN2A* inactivation via promoter methylation, intragenic mutations, homozygous deletion, or LOH with subsequent loss of the other allele, leads to inappropriate cell cycle progression and unregulated cell proliferation and growth. Inactivation is an intermediate event in PDAC progression model, being detected in PanIN2.

_TP53_ Alterations in PanCa

The *TP53* tumor suppressor is inactivated in 50–75 % of PDACs. Located on chromosome 17, *TP53* encodes the p53 transcription factor that controls several

genes involved in cell cycle progression, specifically, G2-M phase arrest and G1-S checkpoint. P53 also controls DNA repair and cell death processes. In cells with severe DNA damage, p53 inhibits cell cycle to allow for possible repair before cycle progression, else it will induce expression of genes involved in cell death (its cell cycle checkpoint functions). Inactivating intragenic mutations causes defective p53 protein production that loses its functional interactions with DNA and hence loss of cell cycle control. Thus, expansion of clones of cells with genomic instability occurs with increasing propensity for cancer formation.

***SMAD4/DPC4* Alterations in PanCa**

Also known as deleted in pancreatic cancer locus 4 (*DPC4*), *SMAD4* is located on chromosome 18q and is inactivated by homozygous deletions and intragenic mutations with loss of the second allele, in as many as 55 % of PDACs. The encoded protein, SMAD4, plays an important role in the TGFβ signaling pathway that functions to inhibit cell growth. The interaction of TGFβ with its receptor (type I and type II serine/threonine kinase surface receptors) induces receptor dimerization and activation of type I receptor that phosphorylates SMAD2 and SMAD3. Phosphorylated SMAD2/3 complex binds to SMAD4, and they translocate into the nucleus where in association with cofactors induce expression of genes that control cell cycle, growth, and differentiation. Loss of SMAD4 causes loss of TGF-β-mediated cell growth control leading to unregulated cell proliferation. Loss of SMAD4 occurs in late stages of PanCa progression, being observed in PanIN3 and infiltrating adenocarcinomas, and is associated with poor prognosis.

9.4.4 Precursor Lesions of PanCa

Approximately 95 % of PanCas originate from the exocrine pancreas, with the remaining 5 % from the endocrine portion. Cancers arising from the pancreatic duct, pancreatic ductal adenocarcinoma, account for ~80 % of all exocrine PanCas, with the remaining being intraductal papillary mucinous tumors, acinar cell carcinomas, mucinous cystic tumors, and serous cystic tumors. Three histologic types of precursor lesions are described for PanCa: pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN), and mucinous cystic neoplasm (MCN) (Table 9.2). Many pancreatic cancers, however, develop from PanIN that usually arises from the small terminal pancreatic ducts.

9.4.4.1 PanIN

Based on the degree of cytological and architectural atypia, three types of PanIN are recognized – PanIN1 (A/B), PanIN2, and PanIN3. The genetics of these lesions are fairly well characterized, and their roles in signaling pathways that mediate disease progression are described in the section on progression model.

Table 9.2 Molecular pathology of pancreatic precursor lesions and cancer

Lesion	Altered genes
Precursor lesions	
PanIN1	Telomere shortening (90 %) *KRAS2* mutations (45 %)
PanIN2	*CDKN2A/p16*
PanIN3	*TP53*, *SMAD4/DPC4*, *BRAC2*
IPMN	*KRAS2 (50 %)*, *TP53*, *CDKN2A/p16*
MCN	*KRAS*, *SMAD4*
Pancreatic cancer	
Adenocarcinoma	*CDKN2A/p16*, *SMAD4*, *TP53*
Adenosquamous carcinoma	*CDKN2A/p16*, *SMAD4*, *TP53*, *P63*
Medullary carcinoma	*MLH1*, *MSH2*
Colloid carcinoma	M*UC2*, *CDX2*
Undifferentiated carcinoma	Loss of *CDH1* (*L1CAM*, *Cox2*, *EGFR*)

9.4.4.2 IPMN

IPMNs are macroscopically visible neoplasms that arise in the mucin-producing main pancreatic duct or their branches. They grow and distend the duct and are associated with excessive mucin production. The genetic alterations of IPMN resemble those of PanIN to some degree. About 50 % of low-grade IPMN harbors *KRAS* mutations, and the mutation frequency increases with the degree of dysplasia. High-grade IPMN lesions acquire *TP53* and *CDKN2A* mutations as well. However, unlike PanIN, *SMAD4* mutations and loss of expression are infrequent in IPMN (occurring in just ~3 % of cases), but invasive cancers that arise from IPMN may harbor these mutations. However, inactivating mutations and loss of tumor suppressor gene functions of the serine/threonine kinase *STK11/LKB1* occur in IPMN, and these are not seen in PanIN. Additionally, activating mutations and *AKT* signaling are observed in ~10 % of IPMN.

9.4.4.3 MCN

MCNs are more frequent in women. They are also macroscopically visible but are typically not associated with the ductal system (tend to be intraparenchymal). They have mucinous epithelial lining and ovarian-like stroma. While the molecular pathology is not well established, mutations in *TP53* and *KRAS* are noted with increasing frequencies mirroring increasing dysplasia. Mutations and loss of *SMAD4* are observed in infiltrating MCN.

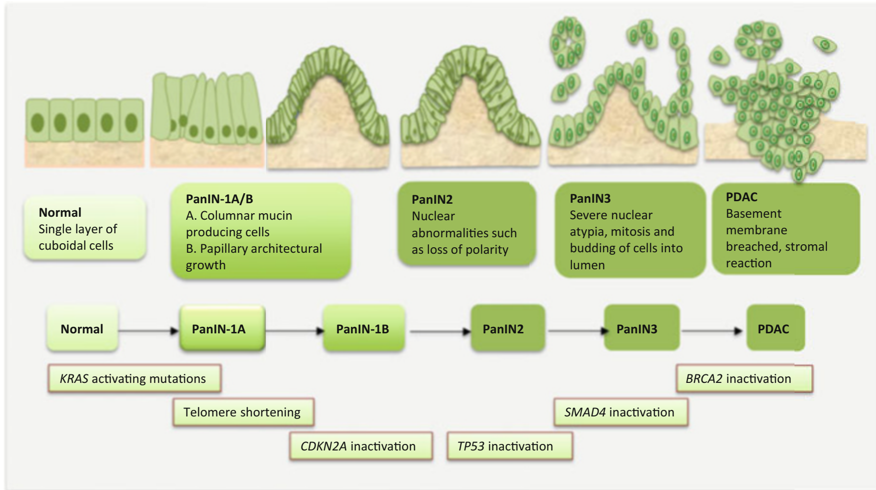

Fig. 9.2 Molecular pathology of multistep pancreatic carcinogenesis

9.4.5 Molecular Progression Model of PanCa

The multistep and field cancerization model of carcinogenesis has been fairly well established for PanCa (Fig. 9.2). While many genetic alterations occur to influence or alter several signaling pathways to drive PanCa progression, three distinct genetic alterations are well proven in pancreatic carcinogenesis. Initial events that occur in PanIN1 are telomere shortening and *KRAS* mutations. Observed mostly in PanIN2 are *CDKN2A* mutations, while late lesions tend to harbor *TP53*, *SMAD4*, and *BRCA2* alterations. Knowledge on the progression of IPMN and MCN through various degrees of dysplasia to cancer is becoming available. This classic model of PDAC development has been supported by genome-wide profiling studies that reveal several mutations relevant to PanCa. The most frequently mutated genes were classified as "high-frequency driver genes," and they included early genes such as *KRAS*, intermediate genes such as *CDKN2A*, and late genes inclusive of *TP53* and *SMAD4*. Complementing the effects of these high-frequency genes are mutations in "low-frequency driver genes" such as *SMARC4A*, *ID1*, *CDH1*, *EPHA3*, *NF1*, *EGFR*, and *FBXW7*.

9.5 Circulating PanCa Biomarkers

The obvious need for noninvasive PanCa biomarkers has led to the discovery of several. The genetic alterations in PanCa (e.g., *CDKN2A* methylation and *KRAS* mutations) are detectable in ctDNA. Also a number of miRNAs show differential levels between patients and control. Traditional serum proteins remain useful, but

proteomic approaches have been extensively employed for discovery studies. The potential diagnostic, prognostic, and predictive roles of circulating PanCa cells require careful study.

9.5.1 Circulating Cell-Free Nucleic Acid Content as PanCa Biomarkers

Circulating cell-free DNA (ccfDNA) in PanCa patients has been analyzed primarily from the standpoint of gene mutations associated with the diagnostic, prognostic, and disease monitoring relevance. However, it also appears the absolute quantitative levels of circulating tumor DNA (ctDNA) have prognostic and treatment monitoring utility in PanCa patients. Elevated plasma DNA > 62 ng/ml was significantly associated with shorter OS ($p = 0.002$), presence of vascular encasement ($p = 0.03$), and metastasis ($p = 0.001$) [4]. Using *KRAS* mutations as a surrogate for ctDNA, 71 % of patients were positive prior to chemotherapy, and many of these patients experienced progressive disease. Importantly, pretreatment ctDNA level was a significant predictor of both PFS and OS. For many of these patients, changes in ctDNA levels corresponded with changes in radiographic imaging and CA19-9 levels on follow-up [5].

Circulating cell-free nucleosomes (ccfnucleosomes) as biomarkers of cancer have been demonstrated and have shown promise for early detection of PanCa. Intact ccfnucleosomes with specific histone modifications and variants were detected using a novel Nucleosome® ELISA platform in circulating blood from PanCa patients [6]. Using multivariate analysis, a panel of five ccfnucleosome biomarkers achieved an AUROCC of 0.95 for PanCa detection. Noteworthy, these patients had resectable tumors. A combination of CA19-9 with a panel of four ccfnucleosome biomarkers achieved the best diagnostic accuracy with AUROCC of 0.98, at a sensitivity of 92 % and specificity of 90 %.

9.5.2 Circulating PanCa Epigenetic Biomarkers

The epigenome is altered in PanCa, with DNA methylation being an early event in PanCa development. CpG island hypermethylation, genomic hypomethylation, as well as miRNA expressional changes underlie pancreatic carcinogenesis. Indeed, multiple genes are silenced or repressed by promoter hypermethylation of CpG islands in ~60 % of PanCas. DNMT1, an enzyme that controls the generational transfer of methylation patterns, is overexpressed in ~80 % of PanCas. Several TSGs, MMR genes, are silenced by promoter hypermethylation in PanCa. Given that PanCa has a favorable cure rate when detected early, such early detection biomarkers assayed in a noninvasive fashion hold tremendous potential for disease

curtailment. However, only a few studies have addressed the clinical utility of circulating PanCa-specific methylation biomarkers.

Preproenkephalin gene promoter methylation was demonstrated in 30 % of plasma samples from PanCa patients, while *CDKN2A* methylated sequences were in 25 % of this cohort [7]. Park et al. observed that methylation of *NPTX2* in plasma could differentiate PanCa patients from those with benign conditions at a sensitivity of 80 % and a specificity of 76 % [8]. Melnikov et al. demonstrated the ability of five methylated genes (*VHL*, *CCND2*, *SOCS1*, *THBS2*, and *PLAU*) in stratifying cancer cases from controls at a sensitivity and specificity of 76 % and 59 %, respectively [9]. In a follow-up study by this group, microarray was used to analyze ccfDNA, and this enabled the identification of 17-gene promoter methylation of relevance to PanCa detection. This gene signature could differentiate chronic pancreatitis patients from controls at a sensitivity of 81.7 % and specific of 78 %. Of even more importance in the detection of PanCa, the performance of these methylated gene panel had a sensitivity of 91.2 %, specificity of 90.8 %, and an overall accuracy of over 90 % in stratifying PanCa patients from those with chronic pancreatitis [10].

9.5.3 Circulating PanCa Genetic Biomarkers

The detection of *KRAS* mutations in circulation of PanCa patients has shown low sensitivity but has proven very specific for cancer detection. Some mutations found in plasma are not associated with PanCa, because circulating *KRAS* mutations are not isolated mutations of PanCa and could also predate the development of cancer. It should however be noted that a single mutation is probably insufficient to cause cancer, and hence such *KRAS*-mutation-positive individuals without cancer ought to be monitored closely for possible early PanCa detection, especially in the presence of risk factors. Indeed, *KRAS* mutations have been detected in plasma of healthy people who were diagnosed with PanCa 5–10 months later.

Mutations in *KRAS* codon 12 have been examined in plasma for their diagnostic and prognostic value in patients who were followed for various clinical outcomes. Detection of mutation in plasma was at 27 % in one series [11]. Plasma *KRAS* mutation was related to tumor size, distant metastasis, and shorter survival time, and this was an independent prognostic factor. Plasma *KRAS* mutant genomes were however not that sensitive (27 %) but very specific (100 %) in detecting cancers among patients with pancreatic masses [11]. Dabritz et al. also analyzed *KRAS* codon 12 mutation in plasma samples, and the frequency of detection was 28 %, with valine mutation being commonly (83 %) detected at this location [12]. In a follow-up study, circulating *KRAS* mutations in plasma were found in 36 % of PanCa patients but not in individuals with pancreatitis. Some patients with low to moderate CA19-9 levels were diagnosed early using plasma *KRAS* mutations (35 % of cases). When combined with CA19-9, plasma *KRAS* mutations achieved a sensitivity of 91 % in the detection of PanCa [13]. Moreover, mutant *KRAS* in

plasma samples corresponded to CT imaging findings in some patients [14]. Plasma *KRAS* codon 12 mutation as well as promoter methylation of *CDKN2A* and *preproenkephalin* (*PPENK*) were examined and data related to smoking status among PanCa patients. *KRAS* mutation was detected at a frequency of 32.5 % compared to promoter methylations of *PPENK* (29.3 %) and *CDKN2A* (24.6 %). However, when considering alterations of at least one of these biomarkers, the detection rate of PanCa was 63 %. Smoking was associated with *KRAS* codon 12 mutations, especially G to A, more so in heavy smokers, but no association was found for promoter methylations and smoking [7].

Circulating tumor DNA as detected using *KRAS* mutation has clinical utility in PanCa patients. *KRAS* somatic mutations (detected at a rate of 29.2 %) and somatic copy number alterations and gene amplifications were detectable by targeted deep sequencing and other genetic approaches [15]. The prognostic relevance of *KRAS* mutation detection in ctDNA is being established. Codon 12 mutations at G12V, G12D, and G12R were detected in 37.3 %, 29.3 %, and 8.0 %, respectively, in tumor tissues, and at 34.6 %, 38.6 %, and 5.3 %, respectively, in corresponding ctDNA, with a concordance of 77.3 % [16]. While *KRAS* mutations in tissue samples had no prognostic prediction, patients with circulating *KRAS* mutations had significantly shorter survival. Earl et al. arrived at a similar conclusion [17]. While the overall detection rate of *KRAS* mutations in circulation was low (26 %), OS was 60 days for *KRAS* mutation-positive compared to 772 days for *KRAS* mutation-negative patients. CTCs detected by CELLSEARCH® system were positive in 20 % of these patients and was equally associated with poor survival. However, compared to ctDNA that was positive in both patients with resectable and advanced diseases, CTCs were mostly detected in patients with metastatic disease.

Detection of other genetic alterations in ctDNA has diagnostic and prognostic associations. A next-generation sequencing approach enabled detection of pancreatobiliary tumor mutations in 90.3 % of ccfDNA that achieved diagnostic sensitivity, specificity, and accuracy of 92.3 %, 100 %, and 97.7 %, respectively. Importantly, these mutations can be detected without the need for knowledge of tumor genotype [18]. A whole gene sequencing of tumor tissue uncovered mutations in *MLL*, *MLL2*, *MLL3*, and *ARID1A* in 20 % of patients. As an early detection biomarker, 43 % of patients with localized tumors had detectable ctDNA at diagnosis. The presence of ctDNA after tumor resection was associated with poor outcome, and relapse, which was detectable 6 months before imaging detection [19].

9.5.4 Circulating PanCa Coding RNA Biomarkers

The circulating transcriptome has not been a focus of PanCa studies. However, Harsha et al. catalogued extensive expression data on pancreatic ductal adenocarcinoma (PDAC) and were able to identify 2516 differentially expressed genes

between cancer and controls. Of these, 930 were detectable in body fluids, and 162 encode secreted proteins. [20]. The largest digital gene expression study of PanCa used serial analysis of gene expression coupled with massively parallel sequencing by synthesis. Five hundred and forty-one (541) genes showed differential expression over tenfold in >90 % of PDAC compared to normal controls. A good proportion of these genes also encode secreted proteins, including S100P that is already implicated in PDAC [21]. The findings from these expressional studies suggest the needed effort to identify the best candidates that can perform optimally for development of a noninvasive screening test.

9.5.5 Circulating PanCa Noncoding RNA Biomarkers

9.5.5.1 Functional Role of PanCa miRNAs

Oncomirs and tumor suppressormirs have been identified in PanCa. Pancreatic cancer oncomirs, which are upregulated in tissue samples, include miR-27a, miR-132, miR-155, miR-194, miR-200b, miR-212, miR-214, miR-220c, miR-310a, miR-421, miR-429, and miR-483-3p. The RB protein controls the cell cycle by sequestering E2F transcription factor. Retinoblastoma is a target of miR-132 and miR-212. By degrading RB, these miRNAs permit uncontrolled cell cycle progression in PDAC cells. Oncogenic PanCa miR-421 and miR-483-3p target *SMAD4*/*DPC4*, an inhibitor of cell growth receptor tyrosine kinase. MiR-27a decreases expression of *SPRY2* leading to increased PDAC cell growth and migration. The EP300 protein suppresses tumor growth and metastasis. MiR-194, miR-200b, miR-220c, and miR-429 are highly expressed in metastatic than nonmetastatic PDAC cells, and EP300 is a target of these PDAC oncomirs. Another overexpressed miRNA in PDAC tissues is miR-155, and this targets and degrades tumor protein 53-induced nuclear protein 1 (TP53INP1) tumor suppressor. The NF-κβ pathway is activated by miR-310a via its repressive activity on NF-κβ-repressing factor (NKRF) in PDAC. As a molecular amplification positive feedback loop, activated NF-κβ signaling induces expression of miR-310a. Finally, miR-214 reduces sensitivity of PDAC cells to gemcitabine and also targets the inhibitor of growth protein 4 (ING4).

Identified miRNAs in PDAC with tumor suppressor functions include miR-15a, miR-34a, miR-96, and miR-375. These miRNAs are generally downregulated in PanCa and hence lose their repressive functions on oncogenes and other positive modulators of cancer growth and survival pathways. MiR-96 directly targets *KRAS*. Being downregulated then enables activation of KRAS/AKT pathway. Similarly, PDK1/AKT pathway activation in PanCa is partly due to decreased expression of miR-375, which targets *PDK1*. MiR-15a targets *FGF-7* and *WNT3A* involved in cancer cell survival. Finally, miR-34a acts through the p53 pathway to suppress tumor growth and hence its downregulation in PanCa.

9.5.5.2 Circulating PanCa miRNA Biomarkers

The diagnostic, prognostic, and predictive value of circulating PanCa miRNAs have been explored by a number of investigators. Wang and colleagues were the first to address the role of circulating miRNAs in PanCa patients, targeting miR-21, miR-155, miR-196a, and miR-210 [22]. While the individual miRNA biomarkers had modest performances with AUROCC of 0.62–0.69, a panel of the four plasma miRNAs achieved a much higher accuracy of 64 % sensitivity and 89 % specificity, with an AUROCC of 0.82. Morimura et al. identified plasma miR-18a as a strong single diagnostic biomarker of PanCa with AUROCC of 0.9369 [23]. Circulating levels of miR-18a and miR-210 are increased in patient samples compared to healthy controls, and miR-18a levels decreased following surgery. In another study by this group, seven miRNAs were potential diagnostic biomarkers of PDAC of which miR-16 and miR-196a were the best performers that could even differentiate patients with PanCa from those with chronic pancreatitis. When used as panel biomarkers together with CA19-9, early stage I PanCa was detected at a high rate of 85.2 % [24]. This group further used deep sequencing of serum samples to identify seven more miRNAs (miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, and miR-191) that were elevated and could differentiate PanCa from healthy controls with AUROCC of 0.992 in the training set and 0.985 in the validation cohort. Importantly, the panel could differentiate PanCa patients from those with chronic pancreatitis, achieving an AUROCC of 0.993. Additionally, as early detection biomarkers, this panel could detect >90 % of stages I and II PanCa patients. Elevated serum miR-21 was an independent predictor of poor survival (HR of 8.77) [25].

The Danish Biomarkers in Patients with Pancreatic Cancer (BIOPAC) study prospectively recruited 409 patients with PanCa, 25 chronic pancreatitis patients, and 312 healthy volunteers [26]. Pretreatment (surgery or chemotherapy) blood samples were collected, processed, and screened for differential miRNA expression. Data analysis involved a discovery, training, and validation cohort. At the discovery phase, which included143 patients, 18 cases with chronic pancreatitis, and 69 healthy controls, 754 miRNAs were examined. Using multivariate analysis, 38 miRNAs were differentially expressed between cancer patients and control (patients with chronic pancreatitis or healthy donors). In a training cohort consisting of 180 cancer patients and 199 healthy volunteers, 19 miRNAs were validated as useful diagnostic biomarkers. These were used to generate two diagnostic models referred to as index I and index II. Index I consisted of four miRNAs, while ten miRNAs defined index II. Index I slightly outperformed CA19.9 in the discovery cohort with AUROCC of 0.88 compared with 0.87 for CA19.9. But CA19.9 was a better biomarker at the validation stage with AUROCC of 0.89 compared to 0.83 for index I. Index II, however, outperformed CA19.9 in both the discovery (AUROCC for index II was 0.92 compared to 0.87 for CA19.9) and training (AUROCC for index II was 0.93 compared to 0.90 for CA19.9) cohorts. But again, CA19.9 achieved a better diagnostic accuracy than index II with AUROCC

of 0.89 compared to 0.81 for index II in the validation cohort. The combination of CA19.9 with either index I or index II as panel biomarkers enhanced early PanCa (stage IA–IIB) detection compared to CA19.9 alone [26].

Some miRNAs can distinguish between cancer patients from those with precursor lesions. MiR-483-3p and miR-21 were significantly higher in PDAC patients than controls and could differentiate PDAC from IPMN patients [27]. High miR-21 was of prognostic value, being significantly associated with advanced stage, lymph node metastasis, and shorter survival. Permuth-Wey et al. [28] found 30 circulating miRNAs that could significantly separate IPMN patients from healthy controls with AUROCC of 0.744. Additionally, a 5-miRNA signature could separate patients with high-grade dysplasia and invasive cancer from those with low- to moderate-grade dysplasia with AUROCC of 0.732.

MiRNAs have been identified that could potentially stratify pancreatic tumors. A multicenter and multistate study design enabled identification of miR-486-5p that could separate PanCa patients from normal controls and patients with chronic pancreatitis with AUROCC of 0.861 and 0.707, respectively [29]. Another miRNA from this study, miR-938, could distinguish PanCa patients from those with chronic pancreatitis, pancreatic neuroectodermal tumors, and other pancreatic tumors with AUROCCs of 0.693, 0.660, and 0.618, respectively.

Some miRNAs may be predictive biomarkers of PanCa. Elevated levels of plasma miR-744 achieved an AUROCC of 0.8307 ($p < 0.0001$) in separating PanCa patients from controls. High circulating levels were independent prognostic factors, being associated with lymph node metastasis and recurrence [30]. MiR-744 may confer resistance to gemcitabine, and high circulating levels in patients on this treatment were associated with poor PFS. Thus, several miRNAs, including miR-18a and miR-196a, demonstrate some clinical potential as PanCa biomarkers.

9.5.6 Circulating PanCa Protein Biomarkers

9.5.6.1 Single Serologic Biomarkers of PanCa

Serum CA19-9 as PanCa Biomarker

Carbohydrate antigen (CA) is a sialylated Lewis blood antigen characterized for uses in cancer management. CA19-9 (sialyl Lewis *a*) was discovered as an antigen that reacted to the monoclonal antibody N19-9, which was initially generated against colon cancer cell lines. It is a carbohydrate antigen secreted by many epithelial cells but found attached to the surfaces of RBCs. Mucins secreted by PanCa cells harbor the oligosaccharide epitope of CA19-9, and this forms the basis for its use in PanCa management. Thus, this oligosaccharide present in sera from PanCa and biliary tract cancer patients has been explored for diagnosis, prognosis, and treatment monitoring. The Lewis antigens are fucosylated by two enzymes: *Le* or fucosyltransferase that produces Lewis *a* and *Se* or fucosyltransferase 2 that

generates Lewis *b* antigens. CA19-9 is primarily fucosylated by *Le*, and this antigen is detectable by the monoclonal antibody against CA19-9. CA19-9 can undergo *Se* fucosylation as well, but the Lewis *b* antigen reacts weakly with this antibody. This observation implies that for positive CA19-9 serology, a person must have at least the Lewis *a* antigen (requires a genotype of Le *a*+/*b*+ or Le *a*+/*b*−). However, 5–10 % of the Caucasian population is Lewis negative (Le *a*−/*b*−) for whom the test is meaningless.

CA19-9 is not a useful diagnostic biomarker for PanCa due to less than optimal specificity (high false-positive rates) and sensitivity. Several patients with benign pancreatobiliary diseases have elevated blood levels. In up to 40 % of patients with chronic pancreatitis, CA19-9 levels are elevated. Up to 10 % of the general population do not express this antigen as well, and just two thirds of those positive for this antigen have surgically resectable disease. This serum biomarker is, however, useful for disease monitoring and has shown promise in diagnosis when combined with other biomarkers. CA19-9 is not sensitive (required sensitivity of >90 %) and specific (required specificity of >90 %) enough for screening purposes. This has been proven by numerous studies including the seminal Japanese study [31]. However, when the cohort of patients is primarily of people with pancreatobiliary disease, the sensitivity of CA19-9 improves slightly. In general, for diagnosis of PanCa, CA19-9 achieves a sensitivity range of 44–90 % and a specificity range of 45–88 % using various cutoff values. The false-positive rate is however as high as 30 % in patients with chronic pancreatitis and biliary diseases [32, 33]

CA19-9 is currently the FDA-approved biomarker for monitoring PanCa treatment outcomes. Multiple studies also indicate that preoperative CA19-9 levels are of no prognostic relevance. Studies have shown that levels >300 U/ml indicate the presence of advanced stage disease. But only a third of this group will have unresectable cancer (advanced stage disease). It is therefore suggested that elevated levels of >130 U/ml in PanCa patients should trigger further evaluation by staging laparoscopy.

Following PanCa resection, postoperative measurement of CA19-9 is useful for predicting disease behavior. Even using different cutoff values, the consensus evidence is that low levels after surgery are associated with improved OS. For instance, using a cutoff value of <180 U/ml, elevated levels post-resection had a hazard ratio of 3.6 [34], and with a lower cutoff of <70 U/ml the hazard ratio was 7.8 [35]. In general, the absence of reduction of CA19-9 to within normal limits (upper limit of 37 U/ml) after complete tumor removal confers dismal outcome with a median survival of <1 year. This finding is important, because even patients with abnormally high preoperative levels (>900 U/ml) who achieve reductions to within the normal range have similar survival outcomes as those with lower preoperative levels [36]. In advanced stage disease, CA19-9 levels still have prognostic value following surgical treatment, chemotherapy, or radiation therapy. Decreases to below 75 % from baseline after treatment are associated with improved survival. Similarly, in metastatic disease, decreased levels after treatment are associated with improved survival. For example, a clearance in CA19-9 levels

of >75 % from baseline was associated with a median survival of 1 year compared to those without such drop [37, 38].

Serum MIC1 as PanCa Biomarker

GDF15, also known as macrophage inhibitory cytokine 1 (MIC1), belongs to the TGFβ superfamily. While initially identified in macrophages, many cancers, including lung, colorectal, and CNS neoplasia, overexpress MIC1, which is also a p53-regulated cytokine. It has pro-apoptotic and antitumor activities especially in early stage cancer, but in advanced stage disease, it may enhance invasiveness and metastasis. The association of MIC1 with PanCa was uncovered by the work of Koopmann and colleagues who showed both overexpression of the message in cell lines and protein in tissues by IHC [39, 40]. A MIC1 ELISA assay was then developed and used to screen serum samples from patients and controls. Of a number of serum biomarkers, MIC1 and CA19-9 were independent predictors of PanCa. This study achieved a sensitivity of 71 % and a specificity of 90 %, which appeared less optimal compared to CA19-9 (sensitivity of 78 %, specificity of 94 %). However, the diagnostic accuracy by ROC analysis indicated MIC1 was superior to CA19-9 when differentiating PanCa patients from controls (AUROCC of 0.99 for MIC1 compared to 0.78 for CA19-9). When the control group consisted of patients with chronic pancreatitis, they perform almost identical (MIC1 AUROCC of 0.81 vs. 0.74 for CA19-9).

Serum CEACAM1 as PanCa Biomarker

Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein-1) carrying Lewis x (also known as CEACAM1) is a member of the Ig superfamily that is expressed and carried on the surfaces of lymphocytes, granulocytes, endothelial, and epithelial cells. A number of cancers including breast, endocrine, and bladder cancers overexpress CEACAM1. Functionally, CEACAM1 controls tumor growth, angiogenesis, and immune modulation. Simeone et al. reported its overexpression in PanCa and developed an ELISA assay for serum measurements [41]. This group observed expression frequencies of 91 % in PanCa, 66 % in chronic pancreatitis, and 24 % in normal control sera. Diagnostic performance was superior to CA19-9 and was enhanced when both were used as a panel.

9.5.6.2 Panel Serologic Biomarkers of PanCa

Several proteomic biomarkers relevant to PanCa have been identified using various proteomic approaches. Many are catalogued and panel combinations tested and validated on independent sample sets (Table 9.3). In general, marker panels have shown potential as screening assays, although a given panel may not be superior to some single biomarkers. There is the need therefore to select the appropriate biomarkers as panel combinations.

A consideration in the use of panels is the set criteria for making a positive call. In general, panels have been combined using an *"or"* connection, which gives a positive result even if only one marker is positive according to its reference cutoff

Table 9.3 Serologic biomarker panels for PanCa

Biomarker panels	Performance
CA19-9, CA242, CA72-4	SN-89.2 SP-62.7
ALCAM, TIMP1, ICAM1, REG1A, REG3, LCN2, IGFBP4	AUROCC-0.96
CA19.9, SAA, HP/HPA1S	SN-81.3 SP-91.3
VNN1, MMP9	SN-95.8 SP-76
CA19-9, ICAM1, OPN	SN-78 SP-94
CA19-9, CEA, TIMP1	SN-71 SP-91
CA19-9, REG1B	AUROCC-0.88
CA19-9, SYNC, REG1B	AUROCC-0.87
CA19-9, AGR2, REG1B	AUROCC-0.87

SN sensitivity (%), *SP* specificity (%), *AUROCC* area under the receiver operating characteristic curve

value. This method will reject only a few samples, especially if many biomarkers are used. This set criterion enhances sensitivity but hampers specificity. Other investigators use the *"and"* connection, which makes a call only when all panel markers are positive in relation to their respective cutoff values. This enhances specificity because of the stringent criteria but at the expense of sensitivity.

In view of the dismal performance of CA19-9 in PanCa screening, panel biomarkers have been examined with some promising discovery results. Brand et al. studied panels using multiplex immunoassay approach [42]. The panel of CA19-9, ICAM1, and OPN gave a sensitivity and specificity of 78 % and 94 %, respectively, in differentiating PanCa from healthy controls. For the patients with nonmalignant pancreatic diseases as controls, a panel of CA19-9, CEA, and TIMP1 gave a sensitivity of 71 % and a specificity of 91 %. Proteomic approach on cell lines and pancreatic juices enabled a candidate list of PanCa biomarkers to be generated. Four of the biomarkers (REG1B, SYCN, ARG2, and LOXL2) were validated in serum samples using ELISA assays. Marker levels were individually higher in cancer patients than controls. While SYCN, REG1B, and ARG2 levels were significantly elevated in cancer patients' sera, CA19-9 had the best performance by ROC analysis (AUROCC of 0.82). Three-marker panel assays, each inclusive of CA19-9, performed better than CA19-9 alone. Importantly, for early stage disease (stage I/II) SYCN, REG1B, and CA19-9 as a panel had the best discriminating ability of cancer from healthy controls (AUROCC of 0.87) and from benign diseases and other cancers (AUROCC of 0.92).

9.5.6.3 Circulating PanCa Protein Spectral Peak Biomarkers

Serum proteomics have enabled the discovery of multiple PanCa biomarkers based on *m*/*z* peaks signatures. Two significantly discriminating circulating peptide peaks at *m*/*z* 3884 and 5959 had a sensitivity of 86.3 % and specificity of 97.6 % in differentiating PanCa from controls. The peak at *m*/*z* 3884 in combination with CEA and CA19-9 had a diagnostic AUROCC of 100 %. Mass *m*/*z* 3884 was identified as platelet factor 4 (PF4) [43]. Another two-peak panel from SELDI-TOF analysis performed at a sensitivity and specificity of 91.6 % in separating cancer from healthy controls and a sensitivity of 90.9 % and specificity of 80 % when chronic pancreatitis patients were the control group [44]. Navaglia et al. explored biomarkers for diabetic-related PanCa. Discriminatory peaks in heterogeneous groups of diabetes patients with or without cancer were at *m*/*z* 1211, 1526, and 3519 [45]. The peak at *m*/*z* 3519, identified as a member of the EGF-like family, was replaceable with CA19-9 in classification algorithms. Diabetics with and without cancer could be differentiated with peaks at *m*/*z* 1211, 1802, 3359, and 7903, as well as CA19-9. With these five biomarkers, 100 % of type 2 diabetics, 97 % of chronic pancreatitis, and 77 % of PanCa patients could be correctly classified. Overall MS features, which achieved an AUROCC of 0.938 vs. 0.883 for CA19-9 alone, enhanced diagnostic accuracy of CA19-9.

9.5.6.4 Circulating PanCa Proteomic Biomarkers

Several groups have used proteomic approaches to identify specific serum proteins of potential utility in pancreatic cancer detection and management. One of the early proteomics studies used a mouse model of PanCa to search for serum biomarkers [46]. Orthotopic nude mice injected with human PanCa cell line were analyzed by SELDI ProteinChip technology. A peak of 11.7 kDa was found to correlate with tumor size and weight and was identified as serum amyloid protein A (SAA). In sera from patients, the levels of SAA correlated with clinical stage and were significantly elevated in comparison to levels in healthy controls and people with pancreatitis. Serum amyloid protein A had a sensitivity of 96.5 % but a low specificity of only 31.9 % for the detection of PanCa. Efforts by Bloomston et al. led to the detection of 154 overexpressed proteins in PanCa compared to controls [47]. In a cross validation study, nine spots discriminated cancer from control individuals at a sensitivity of 100 % and a specificity of 94 %. Noteworthy, fibrinogen gamma was identified as a prominent potential biomarker of PanCa.

Of 24 differentially expressed peaks between cancer and controls, 21 were underexpressed in PanCa patient samples. In a validation test data set, three of the biomarkers had a sensitivity of 83 % and a specificity of 77 % for cancer detection. Apolipoprotein AI, apolipoprotein AII, and transthyretin were all downregulated over twofold in PanCa patient samples [48]. Kojima et al. observed eight peaks from low molecular weight fraction (at *m*/*z* 4470, 4792, 8668, 8704,

8838, 9194, 9713, and 15,958) that differentiated PanCa from healthy controls at a sensitivity of 88 % and a specificity of 93 % and from chronic pancreatitis at a sensitivity of 88 % but a dismal specificity of 30 % [49]. For both controls (healthy controls and chronic pancreatitis patients), the eight biomarkers achieved a specificity of 66 % at the same sensitivity. The most discriminating peak was identified as apolipoprotein CIII (*m*/*z* 9713). This study however uncovered that urinary proteomics was much superior with both sensitivity and specificity of 90 % in differentiating PanCa patients from healthy controls and chronic pancreatitis patients. In another study, SELDI-TOF MS biomarker peaks were first identified from a training set, followed by two independent validations including an ELISA assay. Proteins of interest were identified as apolipoprotein AII and apolipoprotein C1. These two in a panel with CA19-9 had improved diagnostic accuracy.

Several other proteins/peptides of diagnostic relevance have been uncovered in circulation of PanCa patients. Three large mass proteins comprised of α-2-macroglobulin, ceruloplasmin, and complement 3C were overexpressed in PanCa compared to control samples. In this study, the low-mass protein peaks were heterogeneous especially among the cancer patients, but 20 peaks each was uncovered that correlated with cancer and controls [50]. Low molecular weight protein profiling in a training set followed by validation in an independent data set with high-density reverse phase protein microarray unveiled CXC chemokine ligand 7 (CXCL7) to be significantly downregulated in PanCa patient samples. As a single biomarker, CXCL7 had a diagnostic performance with an AUROCC curve of 0.84. CXCL7 was even downregulated in samples from early stage disease (stage I/II) patients. Circulating CXCL7 levels did not correlate with CA19-9, and hence in combination, the two achieved an improved AUROCC curve of 0.961 for PanCa detection [51]. Pancreatic cancer proteomics and validation of selected biomarkers in an independent cohort of cancer, chronic pancreatitis, and non-pancreatic disease patients identified TIMP1 and ICAM1 that outperformed CA19-9 in PanCa detection. Importantly in this study, protein AZGP1 was identified as a biomarker of chronic pancreatitis. If validated, this could help resolve these two difficult to separate conditions [52]. In a follow-up study, SRM-based targeted proteomics platform was used for biomarker identification in plasma samples from cancer, chronic pancreatitis, and healthy control individuals. Three candidate biomarkers, gelsolin, lumican, and TIMP1, were identified that achieved an AUROCC of 0.75 in differentiating cancer patients from controls [53].

Isobaric tags (iTRAQ) labeling for quantification of pooled serum and pancreatic juice samples from a training set and validation cohort led to the identification of elevated expression of complement C5, inter-α-trypsin inhibitor heavy chain H3, α-1-β glycoprotein, and polymeric immunoglobulin receptor (as well as reference proteins REG3A and CA19-9) in PanCa. However, biliary obstruction leading to jaundice interfered with biomarker performances. Biomarker levels were significantly increased by jaundice and had reduced sensitivity in the absence of jaundice. Similarly, biomarkers had reduced specificity between benign jaundice and cancer patients with jaundice. Nonetheless, the combination of all biomarkers had improved performance irrespective of jaundice [54].

Immunoaffinity HPLC column enrichment of low molecular weight proteins followed by 2D DIGE gave differential protein biomarkers that were isolated and subjected to MALDI-TOF MS analysis. Twenty-four proteins were upregulated with 17 downregulated in PanCa. In an independent cohort, apolipoprotein E, α-1 antitrypsin, and inter-α-trypsin inhibitor were upregulated in PanCa [55]. Another immunoaffinity depletion of high molecular weight proteins followed by 2D DIGE revealed eight upregulated and eight downregulated proteins in PanCa compared to controls. Proteins identified include mannose-binding lectin 2 and myosin light chain kinase 2 (a serine/threonine kinase) [56]. Kakisaka et al. also used immunoaffinity depletion enriching for low-abundant proteins that were separated by anion-exchange chromatography and subjected to 2D DIGE [57]. Thirty-three protein spots were differentially expressed between cancer and controls (27 upregulated, six downregulated in cancer). Upregulation of leucine-rich α-2-glycoprotein (LRG) was identified as potential PanCa biomarker. Seven protein spots were differentially expressed between PanCa and healthy controls on 2DE. LC–MS/MS identified two upregulated spots as isoform of alpha-1-antitrypsin (AAT), which is a potential biomarker of PanCa [58]. Roberts et al. used nano-LC–MS/MS to analyze sera for prognostic biomarkers [59]. Alpha-1-antichymotrypsin (AACT) was identified as a potential prognostic biomarker of advanced stage PanCa and is negatively correlated with overall survival of patients.

Sixty-one differentially expressed protein peaks between *m*/*z* 2000 and 30,000 were used to construct multiple classification trees that enabled discrimination of cancer from controls and benign diseases at a high sensitivity of 83.3 % and specificity of 100 %. Six peaks were related to TNM stage, and one peak at *m*/*z* 4016 decreased postoperatively. A peak corresponding to *m*/*z* 28,068 was identified as C14orf166 (regulated mRNA transcription by polymerase II). C14orf166 was upregulated in sera from patients and in cancerous tissues [60].

9.5.7 Circulating PanCa Metabolomic Biomarkers

Metabolome alterations in circulation of PanCa patients have been evaluated. The diagnostic performance shows some promise. Some deregulated metabolites have been identified as well. A significantly higher levels of isoleucine, triglyceride, leucine, and creatinine and an equally significantly lower levels of 3-hydroxybutyrate, 3-hydroxyisovalerate, lactate, and trimethylamine-*N*-oxide were found in sera from PanCa patients compared to that of healthy controls [61]. Serum levels of glutamate and glucose were elevated on multivariate analysis in PanCa patients, while creatinine and glutamine were high in sera from patients with benign conditions. This metabolomic profile of PanCa patients achieved a diagnostic AUROCC of 0.8372 [62]. Richie et al. revealed significant reductions in the serum levels of metabolites that were associated with five systems in PanCa patients compared to controls (all $p < 0.000025$) [63]. These systems were (i) -36-carbon ultra-long-chain fatty acids; (ii) three choline-related systems including

phosphatidylcholines, lysophosphatidylcholines, and sphingomyelins; and (iii) vinyl ether-containing plasmalogen ethanolamines. In this analysis, selected metabolites were able to achieve an AUROCC range of 0.93–0.97 for PanCa detection. A specific biomarker set, PC-594, belonging to the ultra-long-chain fatty acid system, was validated in US Caucasians and achieved an AUROCC of 0.97 [63]. In another study, a diagnostic model was constructed with serum metabolite profile. This model achieved a sensitivity of 86.0 % and a specificity of 88.1 % in a training set and 71.4 % and 78.1 % in the validation cohort, which included patients with resectable tumors [64].

9.5.8 Circulating PanCa Cells

Circulating PanCa cells (CPanCaCs) are being explored for the diagnosis and management of patients. While not extensively studied compared to other solid tumors such as breast and colorectal cancer, CPanCaC enumeration and characterization are potentially of equal value in patient management.

9.5.8.1 Exploratory Diagnostic Potential of CPanCaCs

As a proof of principle, a number of studies have used various CTC enrichment and detection strategies for CPanCaC studies. Detection rate varies considerably partly due to methodological and technical issues, as well as the obvious confounding variable of clinicopathologic factors. For instance, CPanCaC detection by nested PCR targeting *KRT20* mRNA achieved a sensitivity of 79 % [65] and 84 % [66] in two independent studies. Detection rates for other targeted transcripts in the Zhou et al. [66] study of the same samples were *hTERT* (100 %), *C-MET* (80 %), and *CEA* (80 %). The CPanCaC detection rate of 37.5 % by the CELLSEARCH® method [67] was much lower than CTC chip [67, 68] that had sensitivities of up to 100 % and with detection of 9–831 CPanCaCs/ml of blood [67, 68]. Immunohistochemistry without CPanCaC enrichment had a detection rate of ~50 % [69], but following negative selection, 80.5 % of pretreatment samples were positive for CPanCaCs [70]. Therapy, however, decreased the detection rate to just 29.3 % of samples, suggestive of treatment response or failures in these patients [70].

9.5.8.2 Prognostic Potential of CPanCaCs

CPanCaC characterization has prognostic utility as demonstrated by a number of studies. Many investigators have explored the clinical importance of CPanCaCs in patients, mostly by PCR-based approaches targeting various transcripts in patient samples.

Soeth et al. used the detection of *KRT20* transcripts to characterize CPanCaCs and DTCs in preoperative stage I–IV PanCa patients and to assess their prognostic value [71]. CPanCaCs were detected at a rate of 33.8 %. However, in univariate survival analysis, a significant association was observed between CPanCaCs and OS. The mean survival time was significantly shorter in *KRT20*-positive (17.9 months) than *KRT20*-negative (26.1 months) patients [71]. CPanCaCs were positive by *KRT19* PCR in 64 % of patients [72]. Postoperative CPanCaCs detected 1 and 10 days were lower than preoperative levels. While this study failed to reveal significant survival benefit, there was a trend toward association of *KRT19*-positive CPanCaCs and worse outcome. Nested PCR targeting *CEA* mRNA was used for CPanCaC detection in enriched blood samples from PanCa patients, of whom 38 % had received curative-intent surgery. *CEA* mRNA positivity was significantly associated with disease recurrence ($p < 0.0001$), and this detection preceded or coincided with radiologic diagnosis of recurrence in these patients [73]. Following immunomagnetic enrichment, CPanCaCs were detected by PCR targeting transcripts of *KRT19*, *MUC1*, *EpCAM*, *CEACAM5*, and *BIRC5*. Pretreatment samples were positive for at least one marker in 47.1 % of patients, and this was significantly associated with shorter PFS (66 vs. 138 days, $p = 0.01$) [74].

The CELLSEARCH® technology detected CPanCaCs in 42 % of patients [75]. However, the presence of CPanCaCs was significantly associated with decreased median OS. About 30 % of PanCa patients present with locally advanced disease at diagnosis. The prognostic potential of CPanCaCs in this cohort of patients was explored. A subgroup of this international multicenter study (LAP 07 trial) had CPanCaCs enumerated and characterized by CELLSEARCH® method prior to and 2 months after chemotherapy. CPanCaCs were detected in a very small proportion (11 %) of these patients (5 % before treatment and 9 % 2 months after chemotherapy). In multivariate analysis, however, the presence of CPanCaCs was associated with poor tumor differentiation and shorter OS (RR of 2.5, $p = 0.01$). Though the detection rate was low in this cohort of 79 patients, the need to explore the prognostic values of CPanCaCs in locally advanced PanCa is indicated [76].

CPanCaCs were detected at a higher rate in patients with unresectable cancer (33 %) compared to those with resectable disease (9 %). While there was a trend toward CPanCaC detection and disease progression, it was insignificant ($p = 0.08$) [77]. Khoja et al. compared two different platforms, the ISET and CELLSEARCH®, in CPanCaC enumeration [78]. As expected, ISET detected more CPanCaCs than CELLSEARCH® (because ISET enriches for CTCs based on size-dependent membrane filtration compared with marker dependent by CELLSEARCH®). But CPanCaC enumeration demonstrated a nonsignificant trend toward decreased PFS and OS.

While no firm conclusions can be drawn, the data so far is promising, suggesting the need for further evaluation of CPanCaCs in PanCa management.

9.5.8.3 Predictive Potential of CPanCaCs

Torphy et al. evaluated the predictive use of CPanCaCs by targeting patient-derived *KRAS* G12 V mutant xenographs (PDX) mouse models of PDAC [79]. Mice were randomized to receive PI3K inhibitor, BKM120, or vehicle, and CPanCaCs were enumerated before and after treatment using microfluidic chip technology. Median CPanCaC counts significantly decreased in the mice following treatment (from 26.61 to 2.21 CPanCaCs/250 uL of blood, $p = 0.0207$), while mice on placebo (vehicle only) did not show significant reduction in CPanCaCs (23.26–11.89 CPanCaCs/250 uL of blood, $p = 0.8081$). In treated mice, reduced CPanCaC counts were associated with decreased tumor growth.

9.5.8.4 Surgery-Induced Release of CPanCaCs

PCR targeting of *EPCAM* mRNA in preoperative, perioperative, and postoperative samples revealed increased frequency of CPanCaC detection immediately after surgery but returned to preoperative levels just a day after surgery (preoperative rate was 25 %, immediately postoperative rate was 67.5 %, and 6 weeks postoperative rate was 23.5 %) [80]. In a pilot study, two different procedures, standard pancreaticoduodenectomy (ST-PD) and no-touch isolation PD (NT-PD), were used with intraoperative sampling of portal venous blood for CPanCaC detection before and immediately after surgery. CPanCaCs were increased in 83 % of patients who received ST-PD compared to 0 % of NT-PD [81]. In both the Sergeant et al. and Gall et al. studies, no survival benefit was demonstrated, but the absence of CPanCaCs with the novel NT-PD technique warrants further exploration because the biologic fate of induced CTC release is not established (putatively these cannot form metastatic deposits due to absence of the EMT process).

9.6 Summary

- PanCa is associated with a high case fatality because of late disease presentation associated with lack of effective treatment regimens for advanced stage disease.
- The late diagnosis is partly due to lack of noninvasive screening tests for all at risk individuals (>age 30).
- Screening, mainly by invasive imaging modalities, is recommended but only for people suspected to have hereditary risk factors such as *BRCA2* mutation carriers.
- However, only ~10 % of all PanCas are hereditary, with the majority being sporadic diseases.
- The molecular pathology of PanCa includes alterations in *KRAS*, *CDKN2A*, *TP53*, and *SMAD4*.

- The majority of PanCas develop from precursor lesions, mostly PanIN, in a multistep molecular progression fashion.
- The epigenetic (e.g., *CDKN2A* methylation) and genetic (e.g., *KRAS* mutation) alterations in PanCa have a noninvasive application in patient management. For example, plasma *KRAS* mutations are associated with prognostic variables.
- MiRNAs are also noninvasive PanCa biomarkers worthy of exploration, validation, and product development.
- Serum proteins, especially panel biomarkers are promising.
- The plethora of novel serum proteins and peptides identified through proteomic efforts requires further confirmatory studies.
- The prognostic and predictive roles of CPanCaCs have been explored with some success.
- PanCa biomarkers in circulation hold tremendous promise for early detection and clinical management of established disease.

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Chapter 10
Renal Cell Carcinoma Biomarkers in Circulation

Key Topics

- Molecular pathology of renal cell carcinoma (RCC)
- Circulating cell-free nucleic acid content as RCC biomarkers
- Circulating RCC miRNA biomarkers
- Circulating cytokine and angiogenic factors as RCC biomarkers
- Circulating RCC cells
- Circulating endothelial and hematopoietic lineage cells as RCC biomarkers

Key Points

- The incidence of RCC is on the rise due to increasing prevalence of risk factors. Metastatic RCC is almost an incurable disease, and yet 25–30 % of all presenting cases have some aspects of metastasis. Noninvasive screening biomarkers are key to primary and secondary prevention.
- While not validated as circulating RCC biomarker, ccfDNA has been extensively investigated in RCC. Of interest, the facile detection should make tracking ctDNA clinically applicable in patient management. Such circulating epigenetic and genetic profiles of RCC are useful in clinical trials as well.
- Circulating RCC cell detection has been technically challenging. However, the angiogenic nature of RCC leads to increased circulating cytokine and angiogenic factors as well as endothelial and hematopoietic lineage cells in RCC patients.

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10.1 Introduction

Renal cell carcinoma (RCC), the commonest variety of renal cancers, still poses a challenge to oncologists, because metastatic RCC (mRCC) is inevitably a fatal disease. Globally, RCC is about the 12th most diagnosed cancer, matching that of pancreatic cancer. The 2012 global estimated incidence and mortality were 337,800 and 143,406, respectively. In the United States, over 62,700 new cases and 14,240 deaths are expected in 2016. There are geographic variations in the epidemiology of RCC. Age-standardized ratio puts the Czech Republic, Lithuania, Slovakia, and the United States among the countries with the highest incidences, while the Netherlands and Iceland have the lowest rates. While these statistics may not seem alarming, the problem is that RCC incidence has been on the rise since the early 1990s, probably due to enhanced detection by imaging. Similarly, some of the known risk factors are on the rise as well, suggesting the incidence of RCC may mirror these rising risk factors in the future. While the incidence tends to be low in the resource-poor regions of the world, mortality is very high due to late presentation and ineffective management (form lack of resources).

There are myriads of risk factors for RCC. Age is a risk factor for RCC, because it is most commonly diagnosed in people over 64 years of age. It is also racially associated, at least in the United States, being more common in African-Americans and American Indians than the general US population. Dialysis, high blood pressure, and associated administration of diuretics elevate the risk for RCC. Modifiable risks are smoking, obesity, and occupational exposure to substances such as cadmium, herbicides, and organic solvents, especially trichloroethylene. Some genetic diseases elevate the risks for RCC as well. These genetic factors include von Hippel–Lindau (VHL) disease (with *VHL* mutations), hereditary papillary and renal cell carcinoma (HPRCC – with mutations in *MET*), hereditary leiomyoma, and renal cell carcinoma (HLRCC – with mutations in fumarate hydratase, *SDHB*).

The dismal outcome of RCC is due to late diagnosis. There are currently no screening recommendations for RCC, and early lesions cannot be detected by palpation. While imaging can detect small tumors, they are inaccurate at differentiating between benign and malignant lesions. Thus, biomarkers representative of renal tumor biology and hence specific to RCC behavior are needed for accurate early detection, classification, staging, prognosis, and treatment predictions. There are such biomarkers available awaiting validation and translation. However, obtaining tumor tissue for biomarker analyses is invasive and nonrepresentative of tumor heterogeneity. Thus, assaying such biomarkers in body fluids (urine and blood) offers a much better alternative and advancement in renal oncology.

10.2 Molecular Pathology of RCC

Many (>90 %) cancers of the kidney are of epithelial cell origin (RCC), of which the most frequent (~75 % of cases) are clear cell RCC (ccRCC), followed by papillary RCC (~15 %), and then chromophobe RCC (~5 %). Rare RCC subtypes include collecting duct RCC (~1 %), with the remaining that constitutes <1 % being medullary, mucinous, tubular, spindle cell, Xp11 translocation RCCs, and carcinoma associated with neuroblastoma (Fig. 10.1). These subtypes have different molecular pathology and hence prognosis and targeted therapeutic responses. Oncocytomas are benign renal cell tumors that may not easily be differentiated from RCC on clinical or radiographic evaluations. Hence circulating biomarker studies must await correct histopathologic diagnosis to avoid inclusion of these tumors as malignant renal cancers. The various subtypes also have distinct modes of spread. Clear cell RCC has the fastest growth rate and spreads outside the renal capsule through the vasculature to the lung, liver, bone, and brain. Papillary RCC spreads mostly to lymph nodes, while chromophobe tumors have a higher propensity to spread to the liver than ccRCC.

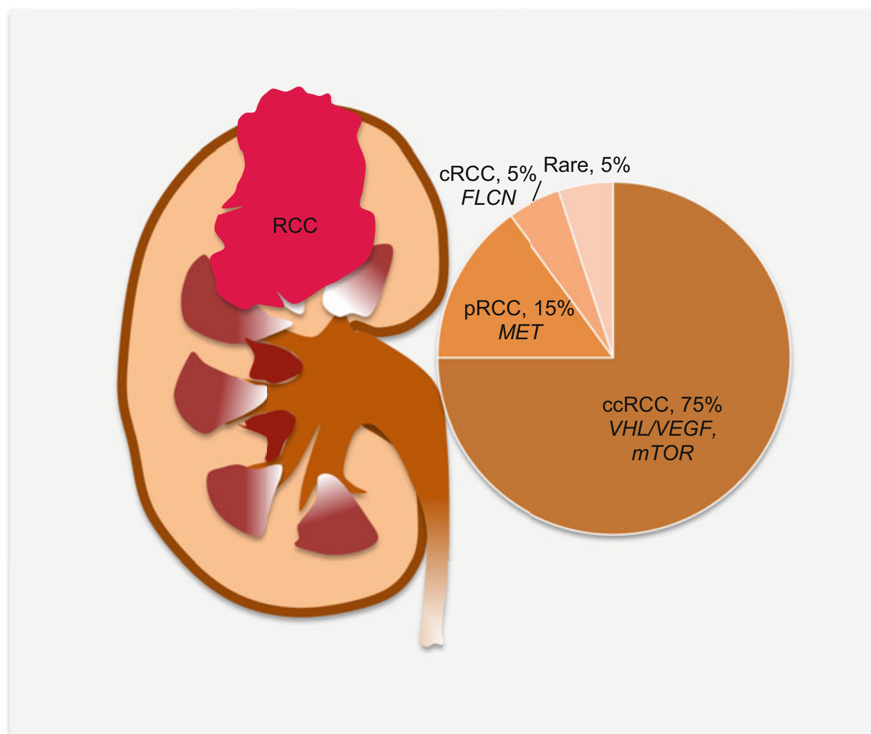

Fig. 10.1 Frequencies of the subtypes of RCC and the commonly associated molecular pathology. *ccRCC* clear cell RCC, *pRCC* papillary RCC, *cRCC* chromophobe RCC

Many RCCs are sporadic; however, ~4 % are associated with hereditary cancer syndromes. Identified are hereditary papillary RCC (type 1 pRCC) and hereditary leiomyosarcoma and RCC (HLRCC; type 2 pRCC). Similar to CRC, these hereditary forms helped inform the molecular underpinnings of sporadic RCC. Indeed, similar molecular pathology is demonstrated for both hereditary and sporadic RCCs, such that it even becomes impossible to separate them based on molecular profiling alone.

10.2.1 Genetics of RCC

The established major signaling pathways deregulated in RCC are the VHL tumor suppressor gene/VEGF, MET membrane-bound tyrosine kinase receptor, mTOR, WNT/β-catenin, and growth factor pathways. But, the best understood molecular pathways in RCC have been elucidated primarily in the major subgroup, ccRCC. Two pathways of clinical relevance (because of the presence of approved targeted agents for members of these pathways) are those tumors that rely on the VHL/VEGF signaling axis and those that employ the mTOR pathway. The vast majority of ccRCC harbors epigenetic and genetic inactivation of the *VHL* tumor suppressor gene on chromosome 3p25-26. Indeed, *VHL* inactivation underlies almost all hereditary ccRCC and in ~ 67 % of sporadic cases. Pathophysiologically, loss of *VHL* functions leads to aberrant induction of HIF gene expression and protein accumulation. An active transcription factor is formed through heterodimerization of HIFα (HIF1α, HIF2α, and HIF3α) subunits with HIF1β (ARNT). In the nucleus, these transcription factors then interact with target gene promoters to induce their expression. Primarily, these are genes induced under hypoxic conditions, which normally promote tumor growth and survival. Additionally induced genes include those for tumor angiogenesis (mostly by HIF2α), glycolytic metabolism (mostly by HIF1α), and cell growth and proliferation.

Different gene alterations cause papillary RCC. The hepatocyte growth factor (HGF) receptor tyrosine kinase, *MET*, is deregulated through mutations and aneuploidy (e.g., chromosome 7 trisomy) leading to increased expression of activated MET in a vast majority of HPRCC and ~75 % of sporadic pRCC. Similarly, HPRCC is caused by germline mutations in *MET*. Mutations in fumarate hydratase, the TCA cycle gene, are linked to HLRCC or type 2 pRCC, and finally mutations in folliculin (FLCN) in Birt–Hogg–Dubé syndrome underlie the rare chromophobe RCC.

10.2.2 Molecular Subtypes of RCC

Molecular subtyping has been successful for some cancers such as breast cancer, and this helps guide efficient targeted therapy. Accurate subtyping of the

heterogeneous RCCs should improve patient stratification for the several currently available targeted therapies. Some progress has been achieved with protein biomarkers, which associate carbonic anhydrase 9 (CA9) with ccRCC and cytokeratin 7 (CK7) with pRCC. Protein biomarker panels have also proved useful in subgrouping the various RCCs. Multiple gene expression studies further suggest that ccRCC has distinct subtypes (ccA and ccB) with prognostic relevance. But gene expression signatures have failed to reproducibly subclassify all RCCs. Also recognized are The Cancer Genome Atlas (TCGA) subtypes of ccRCC and pRCC associated with overall patient survival.

10.2.3 Personalized Oncology in RCC

The use of each patient's tumor molecular genetic profile to tailor or customize management is paramount to successful management of RCC, given its molecular pathologic heterogeneity. Pharmaceutical agents targeting the VEGF and mTOR pathways have been developed. Sunitinib (Sutent, Pfizer), sorafenib (Nexavar, Bayer/Onyx), pazopanib (Votrient, GlaxoSmithKline), and axitinib (Inlyta, Pfizer) are TKIs that target VEGFRs, bevacizumab (Avastin, Genentech/Roche) targets the VEGF ligand, while everolimus (Afinitor, Novartis) and temsirolimus (Torisel, Wyeth/Pfizer) interrupt mTOR pathway by blocking the activity of mTORC1.

Circulating RCC signatures have emerged as potential predictors of patients who will benefit from specific targeted therapies (Table 10.1). In metastatic RCC in particular, obtaining tissue biopsy samples from frail patients may not be the optimal sample source, making the minimally invasive sampling of blood desirable. Not only does this represent primary tumor tissue, it equally harbors cells from metastatic deposits, with possible altered genetic signatures. Moreover, blood can easily be sampled serially while the patient is on treatment to monitor progress and treatment decision-making should resistant clones evolve.

Table 10.1 Targeted therapy in RCC

Agent	Target	Clinical indication
Sunitinib	VEGFR	First-line favorable-intermediate risk
Pazopanib	VEGFR	First-line favorable-intermediate risk
Bevacizumab	VEGF	First-line favorable-intermediate risk
Sorafenib	VEGFR	Second line
Axitinib	VEGFR	Second line
Temsirolimus	mTORC1	First-line poor risk
Everolimus	mTORC1	Second line

10.3 Circulating RCC Biomarkers

Circulating RCC biomolecules have been useful as diagnostic, prognostic, and predictive biomarkers. While all biomarkers have been investigated, the clinical potential of cytokine and angiogenic factors is noteworthy. Circulating RCC cells have been difficult to characterize, but circulating endothelial and hematopoietic lineage cells are promising biomarkers of RCC.

10.3.1 Circulating Cell-Free Nucleic Acid Content as RCC Biomarkers

Some attempts are made on evaluating the diagnostic, prognostic, and predictive role of ccfDNA levels in patients with RCC. DNA integrity assay analysis suggests RCC patients harbor larger necrotic genomic fragments than healthy control individuals, whose circulating DNA is derived mostly from smaller fragmented apoptotic DNA. Gang et al. targeted amplification of *GAPDH* in fragments comprised of 109, 192, 397, and 456 bp in sera to assess DNA integrity in RCC patients [1]. Age and gender were non-confounding variables in both cancer and control groups. For the tumor group, DNA integrity index (DII) had no association with tumor grade. But there was correlation with tumor stage and size. The larger fragments (397 and 456) were undetectable in controls. However, fragment 397 was detectable in 68.2 % of preoperative compared to 31.8 % of postoperative samples, while fragment 456 was detectable in 81.8 % of preoperative compared to 13.6 % of postoperative samples. These findings suggest the tumor cell origin of these ccfDNA fragments. Noteworthy, all samples contained the smaller 109 bp fragment, which is an indication that some ccfDNA in cancer patients are of apoptotic origin (especially in advanced stage cancers). Fragment 397 was detected in 96.2 %, while 456 was observed in 88.5 % of cancer patient samples, suggestive of some diagnostic potential. Hauser et al. used quantitative RT-PCR targeting two fragments of *ACTB* in 35 RCC patient and 54 healthy control samples [2]. ACTB-106 measured smaller apoptotic fragments, while ACTB-384 detected larger fragments possibly from necrotic cancer cells. The ratio, ACTB-384/ACTB-106, measured the levels of DNA fragmentation. The absolute concentrations of both DNA fragments were significantly higher in cancer patients than controls ($p = 0.0003$ for ACTB-384, and $p = 0.003$ for ACTB-106). Expectedly, DII was increased in cancer patients compared to controls (1.07 vs. 0.72, $p = 0.04$). Both of these studies suggest RCC patients harbor circulating DNA from necrotic cells or other modes of release and less so from apoptotic source.

Other studies have addressed the role of ccfDNA in predicting therapeutic response and postoperative recurrence in ccRCC patients. Feng and colleagues quantified ccfDNA as a therapeutic efficacy response biomarker in patients on

sorafenib [3]. Eighteen patients on this therapy had plasma ccfDNA quantified at six different time points (before therapy and at 4, 8, 12, 16, and 24 weeks during treatment). Remission was assessed by CT examination according to RECIST 1.1. Patients in remission or with stable disease had significantly lower plasma ccfDNA from weeks 8 to 24 than patients with progressive disease. Additionally, higher plasma ccfDNA predicted poor outcomes. Using ccfDNA levels at 8 weeks of treatment as a predictor of progressive disease, this assay achieved a sensitivity of 66.7 % and a specificity of 100 %. In order to predict postoperative outcome, ccfDNA was quantified in plasma from 92 ccRCC patients before and after surgery. An increase in ccfDNA was observed in patients with metastatic disease compared to those with localized cancer. Patients with metastatic ccRCC had significantly higher pretreatment ccfDNA (6.04 $\pm$ 0.72) than those with localized disease (5.29 $\pm$ 0.53, $p = 0.017$). As will be expected, the levels in cancer patients were much higher than control healthy individuals (0.65 $\pm$ 0.29 $p < 0.001$). Disease recurrence among patients with localized disease is associated with elevated ccfDNA. High pre- and post-surgical ccfDNA levels have in general been associated with disease recurrence.

Circulating mtDNA measurements may help identify people with urologic malignancies. In patients with renal, bladder, and prostate cancers, circulating cell-free mtDNA was amplified targeting two fragments of the *MT-RNR1* (12S rRNA: a smaller 79 bp and a larger 220 bp fragments). The contents of both fragments (defined by copy numbers) were significantly higher in cancer patients than in healthy controls, and this achieved a diagnostic sensitivity and specificity of 84 % and 97 %, respectively, for the detection of these malignancies. The copy numbers of both fragments were highest in bladder cancer, followed by RCC and then prostate cancer patients. Circulating mtDNA integrity, defined by the ratio of the larger fragment (220 bp) to the smaller fragment (79 bp), was higher in both RCC and bladder cancer patients than healthy controls and men with prostate cancer, and this elevated levels correlated with pathologic state of RCC and with tumor grade in bladder cancer patients [4]. These findings suggest some tumors release more DNA into the circulation than others.

Free circulating RNA levels are also associated with renal cancers. Feng et al. examined circulating RNA levels using serum samples from patients with renal tumors (RCC and oncocytomas) and healthy controls [5]. The mean concentration was significantly higher in RCC patients (1414.19 $\pm$ 91.95 ng/ml) than in those with oncocytomas (560.71 $\pm$ 69.54 ng/m; $p < 0.0001$) and healthy subjects (520.49 $\pm$ 39.75 ng/ml; $p < 0.0001$). The diagnostic AUROCC was 0.956 (95 % CI 0.923–0.989). Serum RNA levels declined in some patients one week after surgery. In another study, this group targeted mRNA of CD133 in peripheral blood as a biomarker of metastasis or predictor of disease recurrence. Blood samples from patients with ccRCC before surgery and healthy controls were analyzed. Patients with metastatic disease had elevated levels of CD133 mRNA (1.546 $\pm$ 0.291) than those with localized disease (1.034 $\pm$ 0.316, $p = 0.022$) and controls (0.042 $\pm$ 0.028, $p = 0.001$) Tumor recurrence was associated with

increases in CD133 mRNA levels and was predictive at 82.6 % sensitivity and 69.8 % specificity [6]. Could these findings indicate the release of RCC stem cells into circulation? This is important because therapeutic targeting of these cells will immensely augment treatment outcomes in such individuals.

10.3.2 Circulating RCC Epigenetic Biomarkers

A number of studies have explored the clinical utility of DNA methylation in circulation of RCC patents. De Martino et al. examined the prognostic performance of both levels of ccfDNA and specific gene methylation [7]. This study involved consecutively collected preoperative serum samples from RCC patients and patients with benign renal tumors. Cell-free DNA analysis targeted *ring finger protein 185* (*RNF-185*), *prostaglandin-endoperoxidase synthase-2* (*PTGS2*), and *CDKN2A*. Additionally, CpG island methylations of *RASSF1A* and *VHL* were assessed. The diagnostic performances of total ccfDNA targeting the specified genes, and methylation of *RASSF1A* and *VHL*, were revealed with AUROCC of 0.755, 0.705, and 0.694, respectively. Noteworthy, *VHL* methylation was associated more with ccRCC than the other subtypes ($p = 0.007$). Total ccfDNA levels were higher in metastatic RCC ($p < 0.001$) and necrotic RCC (0.003) than the other tumors, and these elevated levels conferred poorer disease-specific survival ($p < 0.001$). In multivariate analysis, however, tumor stage, size, grade, and necrosis (SSIGN) score ($p < 0.001$), as well as total ccfDNA ($p = 0.028$), were the significant independent prognostic factors. Another prospective study of serum DNA methylation biomarkers of RCC targeted *APC*, *GSTP1*, *CDKN2A*, *RARβ*, *RASSF1*, *TIMP3*, and *PTGS2* DNA in RCC patients and healthy controls [8]. Methylation frequencies varied from 14.3 % for *CDKN2A*/*ARF* to 54.3 % for *APC* gene, and these correlated with increased tumor stage. Methylation of at least one of these genes was observed in as many as 85.7 % of RCC patients. The methylation of all the genes except *CDKN2A* and *TIMP3* was significantly observed in all patients. While these genes showed very high specificities for RCC detection, the sensitivities were very low. However, as a panel, they performed much better. For example, methylation of *APC*, *PTGS2*, and *GSTP1* achieved a sensitivity of 62.9 % and a specificity of 82 %.

10.3.3 Circulating RCC Genetic Biomarkers

Studies on circulating genetic alterations in RCC have focused mainly on microsatellite alterations (MSAs). Nine polymorphic markers on eight chromosomal regions were used to interrogate MSAs in serum samples from patients with RCC. Allelic imbalance (AI) was detected at a rate of 74 % of these patients. However, by increasing the number of markers to 20, the detection of AI was

increased to 87 %. Most alterations occurred on chromosomes 3p and 5q. The AI was mostly associated with advanced stage carcinomas [9]. In another study, plasma DNA concentration and MSA were examined in patients with ccRCC. Plasma DNA concentration was significantly higher in patients than controls, and the levels decreased after surgery. MSA was found in 75.8 % of tumor tissues and 55.6 % of plasma samples. Increasing plasma DNA concentration and MSAs were predictive biomarkers of recurrence [10].

Because as many as 80 % of ccRCCs have chromosome 3p losses, four polymorphic markers (D3S1307, D3S1560, D3S1289, and D3S1300) on chromosome 3p were used to study tissue and plasma samples from ccRCC patients. Sixty three percent of tissue samples had at least one MSA, of which 35 % were in corresponding plasma, and the tissue and plasma MSAs were identical [11]. Gonzalgo et al. evaluated the prognostic value of MSA using 28 microsatellite markers in a prospective study [12]. Serum, urine, and tissue samples were all examined. Patients had surgery and were followed for 2 years. The frequency of preoperative serum MSA was a significant prognostic indicator of disease recurrence. Preoperative serum MSA is useful in the detection of RCC, and the frequency can identify high-risk groups with possible recurrence after surgery [12]. Thus, MSAs are common in RCC and can be detected in circulation. The potential clinical utility requires further investigation.

10.3.4 Circulating RCC Noncoding RNA Biomarkers

RCC is associated with deregulated expression of miRNAs that may have biologic implications in RCC progression and metastasis. Oncogenic RCC miRNAs include miR-21, miR-23-3p, miR-34, miR-100, miR-142-3p, miR-155, miR-185, miR-210, and miR-224. MiR-21 and miR-23-3p target *PTEN* in RCC and are upregulated. RCC tumor suppressormirs include miR-99a, miR-138, miR-141, miR-143, miR-145, miR-149, miR-192, miR-194, miR-200c, miR-205, miR-215, and miR-1285. Targets of miR-192, miR-195, and miR-215 are *ZEB2* and *MDM2* involved in EMT.

Studies of circulating miRNAs in RCC have been addressed with some potential leads in regard to novel biomarkers. The 2011 study by Wulfken and colleagues uncovered 109 miRNAs elevated in sera from patients with RCC, of which the levels of 36 were concordant with alterations in tissue samples. Validation analysis identified only miR-1233 as having some diagnostic utility with a sensitivity of 77 % but a dismal specificity of 37.6 % [13]. In another study, Redova et al. found 30 miRNAs to be deregulated in sera from patients with RCC, of which 19 were upregulated and 11 were downregulated [14]. In a validation study, only miR-378 (upregulated) and miR-451 (downregulated) were able to separate RCC patients from controls. Used in combination as diagnostic biomarkers, this panel achieved a sensitivity of 81 % and a specificity of 83 %. The elevated HIF associated with *VHL* mutations in RCC controls miR-210 expression. Consistently, the levels of

miR-210 are increased in RCC tissue samples, and this is explored in circulation as well. Sera from RCC patients have elevated levels of miR-210, and these high levels significantly fell a week following nephrectomy ($p = 0.001$). As a diagnostic biomarker, the elevated miR-210 levels had a sensitivity and specificity of 81 % and 79.4 %, respectively [15]. Another study confirmed the elevated levels of serum miR-210 in ccRCC samples compared with paired normal renal tissue. In this cohort, serum levels were equally higher in ccRCC patients than healthy controls ($p = 0.001$). As a diagnostic biomarker, this single serum assay achieved a sensitivity of 65 % and a specificity of 83 % with AUROCC of 0.77 (95 % CI 0.65–0.89) [16]. Serum levels of miR-193a-3p, miR-362, and miR-572 were significantly elevated, while miR-28-5p and miR-378 were decreased in RCC patients compared to controls [17]. The diagnostic AUROCC for this panel of five miRNAs was 0.807 and 0.796, respectively, in training and validation sample sets. Of interest, early stage I patients were detected at a sensitivity of 80 %, specificity of 71 %, and AUROCC of 0.807.

The prognostic role of circulating miR-221 and miR-222 in RCC has been explored. These miRNAs are elevated in RCC tissue samples and are implicated in disease metastasis. The circulating levels are equally high in RCC patients than healthy control individuals ($p = 0.044$), and miR-221 levels were associated with disease metastasis ($p = 0.001$). Higher levels of miR-221 conferred decreased OS (48 vs. 116 months, $p = 0.024$). Cox regression analysis indicated that higher circulating miR-221 was associated with cancer-specific death (HR, 10.7, 95 % CI, 0.13–85.65), $p = 0.026$) [18]. Of interest, other miRNAs have been demonstrated to be elevated in circulation of patients with RCC, but were of no clinical value in this series. These include miR-21, miR-26a-2-3p, miR-191, miR-337-3p, and miR-378. Note, however, that miR-378 is demonstrated to have some diagnostic value. Sensitive technologies and standardized assays will reveal the true value of these elevated miRNAs in circulation of RCC patients. Finally, extracellular vesicles of RCC stem-/tumor-initiating cells are enriched with miR-200c, miR-92, and miR-141 that may shuttle in circulation to participate in preconditioning of metastatic niches [19].

10.3.5 Circulating RCC Protein Biomarkers

Serum proteins have been useful in clinical management of RCC patients. Specifically, the altered levels of cytokine and angiogenic factors (CAFs) have proven useful as prognostic and predictive biomarkers (Fig. 10.2). Adding to this biomarker pool are novel proteins and peak signatures discovered from MS analysis of serum samples.

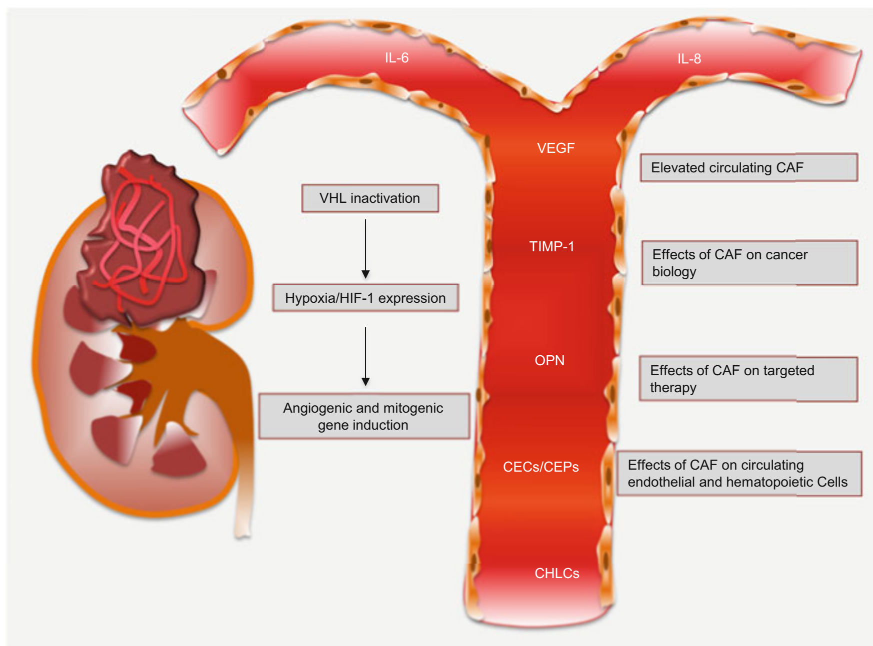

Fig. 10.2 Circulating cytokine and angiogenic factors and their effects in RCC. The differential levels between cancer patients and healthy controls hold biomarker potential

10.3.5.1 Serum Proteins as RCC Biomarkers

RCC Diagnostic Serum Protein Biomarkers

The need for early detection biomarkers for RCC is certainly urgent since the cure rate can be as high as >90 % for pathologic stage T1. To this end, Su Kim and colleagues embarked on analysis of three potential biomarkers (NNMT, LCP1, and NM23A) for early detection of RCC [20]. In this assay, plasma levels of all three biomarkers were significantly elevated in RCC patients compared to healthy controls and people with benign tumors ($p < 0.0001$). Remarkably, in a blinded validation study encompassing 175 controls and 114 patients with diverse subtypes of renal cancer, NNMT as a single biomarker and the three as a panel achieved a diagnostic accuracy of 0.913 and 0.932, respectively. At a set of 90 % specificity, NNMT and the panel had a sensitivity of 71.9 % and 95.7 %, respectively. The PPV (87.2 %) and NPV (97 %) were equally impressive. Noteworthy, these biomarker performances were independent of RCC subtypes.

RCC Prognostic Serum Protein Biomarkers

A number of clinical studies have identified and validated some CAFs as prognostic biomarkers for advanced RCC patients. Multiple clinical trials confirm that advanced RCC patients with high circulating levels of VEGF harbor tumors with high grades and stages and are associated with worse performance status and overall outcome [21]. In the TARGET phase III clinical trial of sorafenib vs. placebo in patients with metastatic RCC, baseline VEGF concentrations were identified in multivariate analysis to be associated with shorter PFS ($p = 0.0231$) and OS ($p = 0.0416$) in the placebo group, as well as shorter OS ($p = 0.0145$) in the treatment arm. Similarly, high baseline VEGF levels were identified in the AVOREN phase III trial of RCC patients on bevacizumab and IFN vs. IFN and placebo to be associated with shorter PFS in both groups [22, 23]. In another analysis of the TARGET trial data, the prognostic relevance of plasma VEGF, sVEGFR-2, TIMP1, sCA9, and RAS p21/RASA1 was evaluated. In multivariate analysis, TIMP1 emerged as a significant prognostic factor ($p = 0.002$) among others such as Memorial Sloan Kettering Cancer Center prognostic score and performance status [21].

Other soluble prognostic biomarkers for the management of patients with metastatic RCC are IL-6 and osteopontin (OPN). High circulating IL-6 correlates with shorter PFS and OS and is an independent prognostic factor in patients on IL-2 or IFN therapy [24]. Similarly, high levels of OPN are associated with metastatic RCC and confer shorter OS [25]. The prognostic role of seven other biomarkers in advanced RCC was examined in the pazopanib phases II and III randomized control trials. VEGF, HGF, OPN, IL-6, IL-8, E-selectin and TIMP1 were analyzed for their prognostic value. Again, high circulating levels of OPN ($p < 0.001$), IL-6 ($p < 0.001$), and IL-8 ($p < 0.002$) were associated with shorter PFS in the placebo group. In a retrospective analysis of the phases II and III pazopanib trial, high circulating levels of IL-8 ($p = 0.006$), OPN ($p = 0.0004$), HGF ($p = 0.010$), and TIMP1 ($p = 0.006$) in patients on pazopanib therapy were associated with shorter PFS than those with low levels (relative to the median value). In the placebo group, determinants of shorter PFS were high circulating levels of OPN ($p < 0.0001$), IL-6 ($p < 0.0001$), and IL-8 ($p = 0.002$) [26]. Serum CA15-3, CA125, and β-2 microglobulin have also been examined for their prognostic use in RCC patients. Cancer-specific survival was low for patients with high levels of these three biomarkers. Multivariate analysis identified only CA15-3 as well as age and visceral metastasis as independent adverse prognostic factors of cancer-specific survival.

RCC Predictive Serum Protein Biomarkers

Inhibitors of angiogenesis have proven useful for RCC management. As example, sorafenib (RAF kinase inhibitor) inhibits VEGFRs 1, 2, and 3, PDGFRβ, Flt-3, C-KIT, and RET. Circulating biomarkers can predict response to this anti-angiogenic therapy. Thus, patients with high circulating levels of VEGF appear to

respond better to VEGFR TKI, sorafenib, than those with lower concentrations. This observation from the TARGET phase III trial should enable personalized medical triaging of patients with advanced RCC [22]. A panel of six biomarkers comprised of VEGF, sVEGFR, sCA9, OPN, collagen IV, and TNF-related apoptosis-inducing ligand were able to determine a select patient population who could benefit, in terms of PFS, from sorafenib alone therapy than its combination with IFN [27]. In the phase III pazopanib vs. placebo study, high circulating IL-6 levels predicted greater benefit from the therapy compared to those on placebo agent ($p = 0.009$). Additionally, it is observed that high IL-6 levels are associated with shorter PFS in the placebo arm, indicating that these patients indeed need pazopanib therapy to reduce their disease burden. High levels of six CAFs, namely, HGF, OPN, VEGF, TIMP1, IL-6, and IL-8, are associated with poor OS. However, these signature biomarkers predict relative OS benefit when treated with pazopanib [27].

Motzer et al. studied SNPs and other serum biomarkers as response biomarkers in patients with advanced RCC on sunitinib therapy [28]. Of all the biomarkers studied, they identified angiopoietin-2 (ANG2), MMP-2, and HIF1α as useful predictive biomarkers. Noteworthy, lower baseline ANG2 and higher baseline MMP-2 significantly correlated with therapeutic response to sunitinib. Other sunitinib predictive biomarkers are baseline IL-8 and VEGFA, as they predict prognosis, and sVEGFR3 levels, which predict efficacy to sunitinib treatment. In multivariate analysis, baseline sVEGFR3 and IL-8 remained as independent predictors of OS in patients on sunitinib-targeted therapy.

In an international phase II randomized trial of patients on mTOR inhibitor, temsirolimus vs. IFN, pre- and post-therapy serum LDH was assayed in 404 poor-risk patients [29]. Baseline LDH levels were elevated in these patients, with mean levels being 1.23 times the upper limit of normal. Patients with increased LDH levels benefited from temsirolimus treatment compared to those on IFN (6.9 vs. 4.2 months, log-rank $p < 0.002$), but patients with normal values did not benefit from this mTOR inhibitor therapy. Patients with high serum LDH > 1 times the upper limit were more likely to die with an HR of 2.01 (95 % CI 1.6–2.6, $p < 0.0001$), when adjusted for known prognostic factors. Two months following treatment revealed increased LDH in both arms of the trial, being 1.7 % for the IFN group and 27 % for patients on mTOR inhibitor. Declining LDH levels with therapy was of significant prognostic relevance.

10.3.5.2 Serum RCC Proteomic Biomarkers

Proteomic approaches have been extensively applied to serum samples for RCC biomarker discovery. A disproportionately large number of protein peak signatures appear discriminatory for RCC. However, the specific proteins and peptides are now being identified.

Won and colleagues used SELDI MS profiling, artificial intelligence, and ProteinChip software to analyze serum samples from RCC and non-RCC urologic disease patients and healthy controls [30]. Peaks identified at *m/z* 3900, 4107, 4153,

5352, and 5987 could accurately differentiate RCC from the control groups. While these peaks could be uncovered with a modified protocol by Engwegen et al., they were of no diagnostic relevance in this cohort of patients [31]. Hara et al. used SELDI-TOF MS to analyze an initial training set of samples and then performed validation studies on blood samples from patients with RCC and healthy controls [32]. Two significantly discriminating peaks at *m/z* 4151 and 8968 were uncovered. Together, these two peaks could differentiate patients with RCC from healthy controls at a sensitivity of 89.5 % and specificity of 80 %. As potential early detection biomarkers, 88.9 % of UICC stage I RCC patients were correctly classified. Of seven differentially expressed peaks, a diagnostic model with peaks at *m/z* 2945.35, 5819.23, 6984.51, and 15,340.8 had a sensitivity of 80 % and specificity of 81.8 % in blind validation study [33]. Chinello et al. assayed sera from ccRCC patients and healthy controls using ClinProt/MALDI-TOF MS technique [34]. Three peptide peaks at *m/z* 1083, 1445, and 6879 could discriminate between cancer and healthy control patients at a sensitivity of 88 % and specificity of 96 % in cross validation analysis. This biomarker cluster was 100 % sensitive for pT1 patients. While this study involved small sample size, it is worthwhile identifying the specific peptides for validation.

Presurgical sera from stages I–IV, postsurgical sera from only stage IV patients, as well as healthy control samples, were subjected to SELDI-TOF MS to validate previously assayed diagnostic biomarkers [31]. In both independent sample sets, only elevated serum amyloid-α cluster could be validated, and this was independent of disease stage or surgical status. Two new peaks at *m/z* 4289 and 8151 were identified as potential diagnostic biomarkers in this cohort [31]. In another study by the same group, 15 protein peaks were significantly different between RCC patients and healthy controls. These peaks were used to generate two classification trees that achieved a sensitivity of 76 % and specificity of 65 % (for classification tree 1) and sensitivity of 83 % and specificity of 82 % (for classification tree 2) in independent samples [35]. Wood et al. obtained preoperative sera from ccRCC patients and healthy controls and subjected these to SELDI-TOF MS and subsequent marker validation by immunoassay [36]. They found several prognostic biomarkers in multivariate analysis, but a fragment of serum amyloid-α at *m/z* 1525 had a hazard ratio of 0.26 ($p = 0.026$). By immunoassay, total SAA was also found to have independent prognostic importance with HR of 2.46 ($p = 0.017$).

Xu et al. analyzed sera from RCC patients and individuals with benign renal masses for diagnostic biomarkers. Peaks at *m/z* 2955.95, 3278.00, and 4657.56 showed diagnostic potential [37]. A double-blind test with a decision tree yielded a sensitivity of 75 % and a specificity of 83.3 %. In 2009, this same group examined sera from RCC, benign renal tumor patients, and healthy controls. A decision tree used in a blinded test achieved a sensitivity of 92 % and a specificity of 95.5 % for the healthy cohort and 35.3 % for patients with benign renal tumors. Peaks at *m/z* 3887.11 and 11,079.8 were potential diagnostic biomarkers of RCC [38]. Further analysis (probably of the same patients) using classification decision tree yielded a sensitivity of 81.8 % and specificity of 100 %. The most highly expressed

circulating protein biomarker in RCC patients was eukaryotic initiation factor 2B delta subunit (eIF2B-δ) [39].

Serum peptidome of RCC was examined by MALDI-TOF LC-MS/MS. Five signal cluster were informative, and several endogenous peptides were identified with SDPR and ZYX levels being low, while SRGN and TMSL3 levels were elevated in RCC patient sera compared to controls [40]. Sera from ccRCC patients and additional paired pre- and post-operative samples as well as healthy controls were subjected to MALDI-TOF MS and ClinProTools software analysis. Twenty-four significantly different protein peaks were observed of which three peaks appeared to be associated with ccRCC because they returned to normal after surgery. These peaks were identified as RNA-binding protein 6 (RBP6) at *m*/*z* 1466.98, tubulin-β chain (TUBB) at *m*/*z* 1618.22, and zinc finger protein 3 (ZFP3) at *m*/*z* 5905.23 [41].

10.3.6 Circulating RCC Cells and Stem Cells

Analyses of circulating renal cell carcinoma cells (CRCCCs) have been fraught with technical challenges, because these cancer cells do not differentiate as epithelial cells and hence do not, in general, express epithelial cell markers such as EpCAM and cytokeratins that have been established in the field of CTC isolation. These cancer cells also do not express specific markers that will enable their differentiation from other blood cells. However, efforts by a few investigators have demonstrated their presence in circulation. Bluemke and colleagues used density gradient and immunomagnetic enrichment techniques followed by cytokeratin 8/18 staining to assess the prognostic role of CRCCCs in 154 RCC patients [42]. This study uncovered CK-positive cells and CK-negative tumor-like large cells that stained for hemalaun blue (Bl+) that also lacked hematopoietic lineage markers, suggestive of being CRCCCs. But the CK-positive cells were detected in just 4.5 % of the patients, consistent with the fact that these cells hardly express any epithelial markers. But the Bl+ cells were detected in as many as 38 % of the patients. The number of detected CRCCCs ranged from 1–51 (mean of six cells), and their presence was associated with lymph node positivity and distant metastasis [42]. Gradilone et al. confirmed the dismal recovery of CRCCCs using epithelial markers [43]. They employed the CELLSEARCH® technology to assay CRCCCs in patients with metastatic RCC. Circulating renal cancer cells were uncovered in just 16 % of the patients. Thus, technology development is critical to elucidate the clinical relevance of CRCCCs.

Bussolati et al. identified a subpopulation of RCC cells that expressed the mesenchymal stem cell marker, CD105 [19]. These cells satisfied the criteria of cancer stem cells, namely, they were able to grow in spheres; had clonogenic ability; expressed stem cells markers such as Oct-3/4, Nanog, and Nestin; and possessed the ability to establish transplantable tumors in vivo. Interestingly, extracellular vesicles derived from these putative RCC stem cells contained transcripts of pro-angiogenic proteins such as VEGF, FGF, MMP-2, MMP9,

angiopoietin-1, and ephrin A3. Additionally, several oncogenic miRNAs were enriched in these RCC stem cell-derived vesicles. While these vesicles are technically difficult to demonstrate in circulation, it is very possible they play a role in pre-metastatic niche formation.

10.3.7 Circulating Endothelial Cells as RCC Biomarkers

The very vasculogenic/angiogenic nature of solid tumors, and in particular RCC, enhances the release and hence circulating levels of endothelial cells in cancer patients, and these may have prognostic relevance [44, 45]. There are two marker-identifiable endothelial cell subtypes:

- Circulating endothelial cells (CECs) are matured endothelial cells that originate from blood vessel walls and can be released as a consequence of vascular injury.
- Circulating endothelial progenitors (CEPs), which are de novo bone marrow-derived cells involved in angiogenesis of the hypoxic cancer cell mass. Circulating endothelial progenitors can be integrated into nascent blood vessels, and they can release pro-angiogenic factors to enhance neovascularization of RCC.

As a consequence of the elevated cytokine and angiogenic factors (CAFs) in RCC patients, there are also altered levels of circulating endothelial and hematopoietic lineage cells [46, 47]. For instance, patients with sporadic RCC have more CECs than healthy control subjects, and the levels of these CECs appear not to differ significantly between patients with localized and those with metastatic disease [48]. Evidence to buttress the renal cancer source of these altered circulating cells was provided by the observation that in patients with *VHL* mutations who develop RCC, CEPs are much higher than in those with mutant *VHL* diseases with different types of cancers [48]. Additional evidence for the elevated CEPs being attributable to RCC biology is the fact that nephrectomy for the treatment of RCC causes decreases in their levels. Whether enumerating these rare cells in individuals with elevated risk factors for developing RCC will enhance early detection is unclear but worth the exploration.

Other findings suggest that CECs and CEPs could be biomarkers for early detection of recurrence in patients with resectable disease who receive surgery. High CEP/CEC ratio was associated with postsurgical disease recurrence. As a prognostic biomarker, it was demonstrated that a progenitor subpopulation identified by specific markers (CD45dim, CD34, and VEGFR-2) was higher in patients who demonstrated worse prognosis [49]. Indeed, CECs and CEPs express VEGFR, and hence their numbers increase in response to the increasing circulating VEGF levels associated with RCC progression. This obviously suggests that effective VEGF inhibition (with VEGFR TKI) should decrease the levels of CECs and CEPs. Consistently, in patients with metastatic RCC on VEGFR TKI therapy, changes in the number of CECs have been observed, and decreases correlate with beneficial PFS [50–52]. The usefulness of enumerating these cells as treatment-

monitoring biomarkers awaits validation studies. Given the entrenched difficulty with CTC analysis of RCC, technologies ought to be developed to further elucidate the role and clinical relevance of CECs/CEPs in RCC.

10.3.8 Circulating Hematopoietic Lineage Cells as RCC Biomarkers

The high levels of circulating CAF (including VEGF, IL-6, and possibly other RCC-specific soluble factors) induce proliferation and hence circulating immature myeloid cells that resemble granulocytes. These cells, characterized by CD11b, CD66b, and VEGFR-1 expression, are able to hamper T cell functions, analogous to myeloid-derived suppressor cells (MDSCs) [53]. The possible use of circulating hematopoietic cells to monitor drug efficacy in mRCC patients on treatment is suggested by a number of studies. Of the VEGFR TKIs, sunitinib appears to have a direct anti-MDSC effect on these circulating cells [54, 55]. Sunitinib also reduces the circulating levels of Treg. These immunomodulatory effects may account in part for the significant association of reduced Treg and myeloid cell subtypes in circulation of mRCC patients on sunitinib and the associated improved PFS and OS [56, 57]. Elevated neutrophil levels in circulation are associated with poor prognosis in patients on VEGFR TKIs [58].

10.4 RCC Extracellular Vesicles

The place for RCC diagnosis using circulating RCC-derived exosomes is yet to be determined; however, the functions of exosomes in RCC biology are being unraveled. Grange et al. demonstrated that microvesicles from CD105-positive renal cancer stem cells stimulate endothelial cell growth and vessel formation, possibly by delivery of pro-angiogenic miRNAs and mRNAs. Moreover, RCC-derived exosomes can induce renal cancer cell proliferation through increased *CCND1* expression, as well as suppression of apoptosis via reduced caspase 3 protein levels. These exosomes may exploit the PI3K/AKT and the MAPK/ERK pathways, because p-Akt and p-ERK1/2 levels were elevated.

10.5 Summary

- The incidence of RCC is on the rise due to increasing prevalence of risk factors.
- Although mortality rates are decreasing (in the resource-rich countries), 25–30 % are late metastatic diseases with dismal outcomes.

- The 5-year survival can be as high as 90 % for localized diseases, but this drops to ~10 % for patients with metastatic disease.
- There are no screening recommendations for RCC probably due to lack of safe and effective noninvasive biomarkers.
- The vast majority of RCCs are sporadic cases, with just a few associated with hereditary syndromes.
- The molecular pathology of RCC is well understood to involve VHL/VEGF, mTOR, and canonical WNT signaling.
- Knowledge on the molecular pathology of RCC has informed the development of effective targeted therapies.
- Circulating biomarkers, especially ccfDNA, epigenetic, and genetic alterations have been explored.
- The angiogenic gene expression profiles of RCC lead to increases in circulating CAFs, which are useful biomarkers.
- Circulating RCC cell detection is technically difficult.
- The increased circulating endothelial and hematopoietic lineage cells are useful RCC biomarkers.

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Chapter 11
Urothelial Bladder Cancer Biomarkers in Circulation

Key Topics

- Molecular pathology of bladder cancer (BlCa)
- Circulating cell-free nucleic acids as BlCa biomarkers
- Circulating BlCa miRNA biomarkers
- Circulating BlCa serum protein biomarkers
- Circulating BlCa cells

Key Points

- Being the most commonly diagnosed urinary tract cancer, BlCa is among the top ten cancers in the world. Early stage low-grade tumors are associated with high 5-year survival rate of >90 %. However, this survival rate is only ~6 % for advanced stage tumors, emphasizing the need for early detection.
- Non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC) may originate from different basal stem cells with distinct genetic lesions. NMIBCs are genetically stable and harbor *FGFR3* mutations, while MIBCs are genetically unstable with *TP53* mutations.
- Potential circulating biomarkers of BlCa are ctDNA, miRNA, serum proteins, and circulating BlCa cells.

11.1 Introduction

Being the most commonly diagnosed cancer of the urinary tract, the 2012 global estimated cases of bladder cancer (BlCa) were 330,380 for men and 99,413 for women. For both sexes, however, BlCa is the ninth in incidence (429,793 for 2012)

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and thirteenth cause of cancer-related mortality (165,084 for 2012). But for men, it is the ninth course of cancer-related deaths (123,051 for 2012). In the United States, the projected 2016 estimates for BlCa are 58,950 and 18,010 for men and women, respectively, while mortality figures stand at 11,820 for men and 4,570 for women. Although the incidence of BlCa has remained quite stable over the past few years, it is among the top ten most commonly diagnosed cancers in the United States.

BlCa is a disease mostly caused by environmental carcinogenic effects on the genome. Tobacco smoke carcinogens cause a substantial (~50 %) number of BlCas. Additionally, occupational chemical exposures such as working in the rubber industry are established risk factors. Because of these risk associations, the prevalence of BlCa is five times higher in men than women. Additionally, this is a disease of the aged, having a median diagnostic age of 65.

Over 90 % of BlCas are urothelial carcinomas. Many (~75 %) of these are diagnosed early when the lesion is confined to the mucosa and submucosa. These tumors are referred to as non-muscle invasive BlCas (NMIBC). The remaining 25 % of BlCas are MIBC, whereby cancer cells will have already infiltrated the muscle wall of the bladder. But ~70 % of these NMIBC patients will develop recurrences within 2 years after treatment as a consequence of *field cancerization (and tumor evolution)*, whereby almost the entire urothelium is exposed to the same carcinogen and hence at risk of developing cancers in the future. BlCas are also dichotomized into low-grade and high-grade tumors based on tumor biology. Low-grade tumors are superficial papillary multifocal tumors that may progress to advanced stage disease. These tend to be associated with good prognosis. High-grade tumors are nodular, and tend to invade early, and hence are associated with adverse outcomes. In general, early stage in situ BlCas or low-grade tumors have a favorable outcome with a 5-year survival rate of about 94–96 %. But this survival rate is markedly reduced to ~6 % in advanced stage disease.

Early stage BlCa is usually asymptomatic. Clinical suspicion is made in patients presenting with blood in the urine (hematuria), increased frequency (polyuria) and urgency of urination, or discomfort/pain on urination. But these symptoms are nonspecific to BlCa and could occur in other bladder conditions such as bladder infection. Thus, a diagnostic work-up including urine cytology, cystoscopy, and possible biopsy of suspicious lesions for histopathologic evaluation is needed. But early BlCa may not shed cancer cells into the urine, thus making urine cytology insensitive (apart from issues with pathologist experience). Cystoscopy is invasive and may not pick up early lesions (without overt histopathologic appearance). Thus, the need for authenticated early detection noninvasive biomarkers should improve the outlook, at least for patients with elevated risk factors. This has primarily been explored in urinary samples for BlCa screening and management, but circulating biomarkers have also been examined and could be useful in disease prognosis and treatment decision-making.

11.2 Molecular Pathology of BlCa

Urothelial BlCas are dichotomized based on histopathologic and molecular characteristics into NMIBC and MIBC. NMIBCs develop from epithelial hyperplastic lesions that progress to low-grade superficial tumors. On the other hand, flat dysplastic lesions that progress to carcinoma in situ give rise to MIBCs. These two pathologic subtypes also have distinct and overlapping molecular pathology. Evidence suggests BlCa develops from two possible BlCa stem (or tumor-initiating) cell lineages. A population of SHH-expressing basal cells with tumor-initiating cell features has been identified in mouse models [1]. Additionally, non-basal tumor-initiating cells are present in the urothelium. Putatively, the duality of this population of cells belies the two pathways, with NMIBC and MIBC emanating, respectively, from the non-basal and basal stem cells niches [2].

The permissive state for progression into either tumor subtype appears to be 9p/9q loss. Further molecular progression of the two pathways can be described simplistically as (Fig. 11.1):

- Superficial NMIBCs are mostly characterized by *FGFR3*, *PIK3CA*, and *HRAS* alterations leading to RAS-MAPK and PI3K pathway activation. These signaling pathways then mediate increased cell proliferation and survival with subsequent development of hyperplasia and low-grade tumors. A significant number (~70 %) will recur, of which a small percentage (10–15 %) may acquire further genetic lesions (e.g., 8p loss, *TP53*, and *RB* mutations) and instabilities to become high-grade MIBC.
- LOH at 9p/9q in association with deregulated cell cycle genes (e.g., *TP53* mutations) characterizes flat dysplastic lesions that often progress to CIS with loss of *RB1* functions. Subsequent gain at 20q, losses at 2q, 8p, and 11q in association with mutations in *PTEN*, *ERBB2*/*HER2*, and *ARID1A* causes progression to invasive disease. Metastasis is mediated by alterations in EMT (e.g., *CDH1*, *ZEB1*, *ZEB2*, and *MMP9*), as well as angiogenic and inflammatory (e.g., *Cox2*, *RHOGDI2*, and *VEGF*) gene expression.

However, this simplistic dichotomized progression model cannot explain the heterogeneity of BlCas. Tumors with features of both types exist. Indeed, within each pathway are different BlCa subtypes, which suggest there are alternative progression pathways. Also low-grade papillary tumors (NMIBC) and their recurrences can display features of high-grade T1 lesions and invasive carcinomas (MIBC). The progression from NMIBC to aspects of MIBC usually involves acquisition of genetic instability (e.g., 8p loss) and deregulated cell cycle control genes (e.g., *CDKN2A*, *TP53*, and *RB* loss of function). A mixed dysplastic/metaplastic lesion progression to papillary high-grade lesion is also possible.

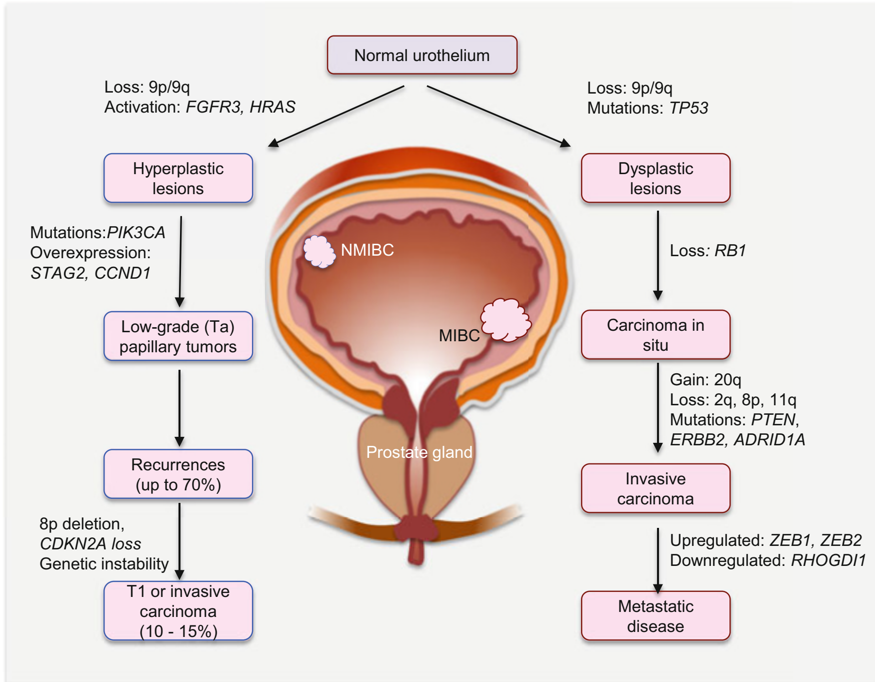

Fig. 11.1 Molecular progression pathways of NMIBC and MIBC. Not shown is the existence of a mixed population of hyperplastic and dysplastic clones that can progress to papillary Ta high-grade tumors

11.2.1 Epigenetic Alterations in BlCa

The epigenome is deregulated in BlCa and has been explored for discovery of noninvasive biomarkers. In addition to miRNA deregulation, aberrant methylation and histone modifications characterize both NMIBC and MIBC. DNA hypomethylation in non-CpG islands is common in NMIBC, while CpG island hypermethylation and gene silencing are a feature of MIBC. Sequencing data has revealed mutations in genes involved in chromatin regulation in ~90 % of MIBC. Commonly mutated and inactivated are *MLL3* involved in histone H3K4 methylation and *KDM6A* that demethylates H3K27 (both create a euchromatin state). Also commonly mutated is a member of the SWI/SNF chromatin-remodeling complex, *ARID1A*.

11.2.2 Chromosomal Alterations in BlCa

While NMIBC tends to display diploid chromosomes indicative of limited genomic instability, MIBC is characterized by aneuploidy, with possible chromothripsis (massive chromosomal damage from a single mutational event) in some cases. Several technical analyses, including FISH, LOH, SNP, CGH, and genome sequence copy number, have uncovered several chromosomal variations in BlCa. Chromosomal deletions, in particular at 9p and 9q and also at 11p, 13q, 14q, and 17p, underlie BlCa development. Chromosome 9 deletions occur in 30–60 % of BlCas and are a major alteration frequently observed in superficial papillary and invasive flat tumors. Deletions at 2q, 5q, and 8p are common in aggressive disease, and gains at 3q, 7p, and 17q, as well as deletions at 9p21, have been identified as clinically relevant diagnostic and prognostic biomarkers. Because of the pathogenic role of 9p/9q loss in BlCa, a number of relevant tumor suppressor genes at these loci have been identified. The following chromosomal locations contain some important genes involved in BlCa: 9p21 (*CDKN2A* and *CDKN2B*), 9q22 (*PTCH*), 9q32–33 (*DBC1*/*BRINP1*), and 9q34 (*TSC1*).

11.2.3 FGFR Alterations in BlCa

FGFR3, and to a lesser extent *FGFR1*, demonstrates alterations in BlCa. FGFR aberrations induce MAPK and PLCγ signaling leading to cellular proliferation, growth, and survival. Hence FGFR activation is mostly associated with NMIBC. The majority (~80 %) of superficial NMIBC harbors activating *FGFR3* point mutations. However, 10–20 % of T1 and invasive BlCas also demonstrate these mutations. While the mechanisms mediating the development of this subset of BlCas (*FGFR3* mutant invasive BlCa) are not well established, these tumors frequently harbor *CDKN2A* homozygous deletions.

The increased FGFR signaling in BlCa can be explained by several mechanisms:

- As noted, activating *FGFR3* mutations occur in a vast majority of these cancers.
- Expression of *FGFR3* and/or its ligand is increased in BlCa. Increased expression of *FGFR3* could be augmented by a SNP in *TACC3*, which is separated from *FGFR3* by 70 kb DNA sequence. This conclusion is because this SNP is associated with BlCa risk and recurrence of Ta disease with *FGFR3* mutations.
- Chromosomal translocation and formation of fusion genes occur in a subset (~5 %) of BlCas. These fusions often involve *FGFR3IIIb* isoform kinase domain in frame to oncoproteins. These oncoproteins include *TACC3* and possibly BAI1-associated protein 2-like 1 (*BAIAP2L1*), leading to increased FGFR3 activation.
- Isoform switches can augment FGFR3 signaling. *FGFR3* exists in different isoforms. Isoform FGFR3IIIb, which is expressed by normal urothelial cells, interacts with FGF1 ligand to control the levels in the urothelium. Another

mechanism of reducing the levels of ligand is the expression and secretion of a spliced variant, *FGFR3Δ8–10*, which binds and sequesters FGFs. In BlCa, a different isoform, FGFR3IIIc, is overexpressed, in association with downregulation of isoforms FGFR3IIIb and FGFR3Δ8–10. Because isoform FGFR3IIIc interacts with several FGF ligands, increased expression can mediate ubiquitous autocrine and paracrine signaling in urothelial cells.

Unlike BlCas with epithelial features, some BlCas overexpress *FGFR1* and *FGF2* ligands, and these tumors demonstrate EMT phenotypes. Of interest, levels of FGFR1β, which has increased sensitivity to FGF1 ligand, and also activates the MAPK and PLCγ pathways through interaction with FGF2, are much higher than FGFR1α spliced variant in these tumors.

11.2.4 MAPK Pathway Alterations in BlCa

The MAPK pathway is activated in BlCa, especially NMIBC. Multiple mechanisms may explain pathway activation. Mutations in RTKs (e.g., *FGFR3*, *RAS*, and *PIK3CA*) are very frequent (>80 %) in early stage (Ta–T1) tumors. While RAS and *FGFR3* mutations are mutually exclusive in BlCa, suggestive of functional redundancy, the disproportionately more *FGFR3*-mutant than *RAS*-mutant BlCas also indicates a possible selective need or oncogenic mechanisms not yet understood. Another poorly understood observation is the distribution of these mutations between NMIBC and MIBC. More NMIBCs harbor *FGFR3* than *RAS* mutations. But *RAS* mutations occur at similar frequencies in both cancer types. However, *FGFR3* and *PIK3CA* mutations tend to occur together in NMIBC and may function to induce both the MAPK and PI3K pathways.

11.2.5 PI3K Pathway Alterations in BlCa

BlCa is also characterized by activation of the PI3K pathway. In ~25 % of NMIBC, the *PIK3CA* is mutated and activate this pathway. Mutations in *PIK3CA* may cooperate with RAS in orchestrating signaling networks in BlCa. Also *PTEN* function is commonly lost in MIBC (LOH is more common than biallelic loss). *PTEN* loss is associated with *TP53* loss, and both may cooperate at some level in the development of invasive disease. Apart from these mutations, several other mechanisms are involved in PI3K pathway activation. EGFR/ERBB1 activates the pathway through RAS. ERBB2/ERBB3 heterodimers may activate the pathway through the interaction of ERBB3 with p110α. Functionally, ERBB activation of the pathway mediates metastasis. Additionally, mutations in *TSC1* and activation of MET and RON/MST1R can activate the PI3K pathway in BlCa. While a role for

FGFR3 is not established in this pathway, increased levels of pAKT are associated with *FGFR3*-mutant tumors [3].

11.2.6 Cell Cycle Alterations in BlCa

The cell cycle is deregulated in BlCa, especially in MIBC, and this is due to myriads of controlling factors including altered signaling pathways and cell cycle regulators. Members of the cell cycle, especially those involved in G1/S phase transition, are abnormally expressed in nearly all MIBCs and are mostly associated with poor prognosis. Established tumor suppressors, including *RB*, *TP53*, and *CDKN2A*, are inactivated in MIBC. On the contrary, genes involved in propelling the cell cycle, including *E2F3*, *MDM2*, *CCND1*, and *CCND3*, are amplified or overexpressed in many MIBC. For example, amplification of *CCND1* occurs in ~20 % of BlCas [4], while The Cancer Genome Atlas data demonstrate inactivation of *TP53* in ~76 % of MIBC. Thus, abnormal cell cycling underlies BlCa proliferation and invasiveness.

11.2.7 Developmental Signaling Pathway Alterations in BlCa

The HH, BMP, and WNT signaling pathways interact with each other and with other oncogenic pathways including MAPK and PI3K in bladder carcinogenesis. While normal urothelial cells express *SHH*, this pathway is abrogated in MIBC. Putatively, loss of SHH signaling blocks stromal cell production of BMP4 and BMP5 needed to drive CIS to invasive cancer. Indeed, The Cancer Genome Atlas data reveal decreased expression of *SHH*, *BMP4*, and *BMP5*, especially in aggressive MIBCs.

Epigenetic and genetic aberrations characterize several members of the WNT pathway in BlCa. The WNT pathway antagonists, *SFRP* and *WIF1*, are silenced via epigenetic mechanisms, while mutations in *CTNNB1* and *APC* are common in MIBC. In mouse models, active β-catenin cooperates with mutant *HRAS* via MAPK pathway activation to drive BlCa development [5]. Additionally, β-catenin on the background of *PTEN* deletion can also induce BlCa development. In human BlCa, *PTEN* downregulation, nuclear β-catenin accumulation, and increased pAKT have been demonstrated [6]. These findings suggest cooperation between the WNT/β-catenin and PI3K pathways in bladder carcinogenesis.

11.3 Circulating BlCa Biomarkers

Circulating biomarkers are not clinically available for BlCa management. However, potential biomarkers are circulating miRNA, serum proteins, and circulating BlCa cells. While they may be of limited use in screening, circulating biomarkers of BlCa will have important utility in disease prognosis, treatment predictions, and monitoring for evolving clones in the metastatic setting.

11.3.1 Circulating BlCa Genetic Biomarkers

A few studies have addressed the possibility of detecting BlCa molecular alterations in circulation. A longitudinal prospective study for mutations in ccfDNA in plasma targeting *TP53* and *KRAS* for prediction of tumor development (possible early detection markers) was conducted where cases were tightly matched to controls, including even geographic residential locations. Of this cohort, 137 subjects developed BlCa, and plasma *KRAS* and *TP53* mutations were detected in five and seven patients, respectively. This longitudinal analysis suggests profiling healthy people noninvasively can identify cancer-risk conferring mutations years before clinical diagnosis of cancer. This may only be useful in the setting of high suspicion or as an adjunct to other diagnostic tests [7].

The genetic instability associated with MIBC has been successfully assayed in circulation of BlCa patients. Seventeen microsatellite markers on nine chromosomal regions were used for the analysis of MSAs in serum samples from patients with BlCa. The frequency of MSA was as high as 84.5 % and 72 % in serum and urine samples, respectively. This study found no association of these alterations with tumor grade or stage [8]. In a follow-up study, fluorescent microsatellite analysis with 17 polymorphic markers covering the nine chromosomal regions was examined in serum samples. Microsatellite alterations were detected at a similar high frequency of 79.3 %. Most alterations occurred on chromosome 8p and in high-grade tumors [9]. This group had demonstrated such findings earlier with a detection rate of 80.3 % [10]. MSA in cell-free serum, plasma, and urine DNA was assessed with six polymorphic markers on three chromosomes. In this series, 72.2 % of BlCa tissue samples harbored MSA, of which detection in at least one of the body fluids was found in 88.5 % of patients. Thus, simultaneous and multiple analysis of blood and urine can increase the detection of circulating BlCa DNA [11]. These proof of principle studies suggest tracking BlCa genetic alterations is feasible and can be applied clinically.

11.3.2 Circulating BlCa Noncoding RNA Biomarkers

Several miRNAs show deregulated expression in BlCa, and their diagnostic potential has been analyzed primarily using urine samples. Oncomirs that are upregulated in BlCa include miR-9, miR-20a, miR-21, miR-96, miR-106b, miR-141, miR-146b, miR-205, miR-183-96-182 cluster, and miR-210. Downregulated are the following tumor suppressor miRs: miR-23b family, miR-29c, miR-124, miR-200 family, miR-214, miR-409, miR-490-5p, and miR-590-3p. These miRNAs target important genes to alter major signaling pathways in cancer. The oncomirs, for instance, target *TP53* (miR-21); *AKT* and *PTEN* (miR-21, miR-205, miR-183-96-182 cluster); *E2F3*, *FGFRL1*, and *HOXA1* (miR-210); and *VEGF* (miR-205), while targets of tumor suppressor miRs include *DNMTs* (miR-29c); *cMET* and *cFOS* (miR-409); *ZEB1*, *ZEB2*, and *ERFFI1* (miR-200 family); *ROCK1* (miR-214); *CTNNB1* (miR-214); and *MMP-2, MMP9,* and *TFAM* (miR-590-3p).

There are several emerging circulating miRNA of possible relevance in BlCa patients. While the studies are preliminary, their potential for patient management has been demonstrated. Plasma levels of miR-148b, miR-200b, miR-487, miR-541, and miR-566 were elevated, while miR-25, miR-33b, miR-92a, miR-92b, and miR-302 were reduced. As diagnostic biomarkers, a logic regression model achieved an overall accuracy of 89 % for BlCa detection and 92 % for discriminating between invasive BlCa and other cancer types [12]. In whole blood from patients with invasive BlCa, miR-26b-5p, miR-144-5p, and 374-5p were significantly elevated compared to controls. MiR-26b-5p could predict the presence of invasive BlCa at a sensitivity and specificity of 65 % and 94 %, respectively [13]. MiRNA profiling of sera from 250 patients and 240 controls identified a panel of six miRNAs (miR-15b-5p, miR-27a-3p, miR-30a-5p, miR-148b-3p, miR-152, and miR-3187-3p) useful in BlCa detection based on a multivariate logistic regression model. This miRNA panel achieved diagnostic sensitivities of 90 %, 84.85 %, and 89.36 %, respectively, for the detection of Ta, T1, and T2–T4 BlCa. In patients with NMIBC, elevated miR-152 and reduced miR-3187-3p levels predicted worse recurrence-free survival. MiR-152 in particular was an independent predictor of tumor recurrence in multivariate Cox regression analysis [14]. Plasma miR-497 (decreased) and miR-663b (increased) were significantly deregulated between cancer patients and controls, with a sensitivity, specificity, and AUROCC of 69.7 %, 69.6 %, and 0.711, respectively, for BlCa detection [15]. Levels of circulating miR-92a, miR-100, and miR-143 were reduced in cancer patients compared to controls. For the detection of NMIBC at defined cutoff values, the sensitivities and specificities were 97.1 % and 76.7 % for miR-92a at a cutoff value of 0.573, 90 % and 66.7 % for miR-100 at a cutoff value of 0.644, and 78.6 % and 93.3 % for miR-143 at a cutoff value of 0.164 [16].

Meta-analyses inclusive of all sample types suggest a role for miRNA in BlCa detection. The study by Chen et al. of miRNA for BlCa detection included 30 studies from ten publications with 1019 patients and 690 controls [17]. The pooled sensitivity, specificity, PLR, NLR, DOR, and SAUROCC were 80 %, 74 %, 3.2,

26 %, 15.20, and 0.85, respectively. This indicates a promise in BlCa detection, especially when used as panels, because multiple miRNAs could achieve a sensitivity, specificity, and SAUROCC of 86 %, 80 %, and 0.913 for diagnosis of BlCa. Moreover, a sensitivity, specificity, and AUROCC of 74 %, 77 %, and 0.84 were achieved for detection of NMIBCa. Another meta-analysis of reports on blood and urine assays included 4558 patients and 4456 control. In this study, the sensitivity, specificity, and AUROCC were 74 %, 78 %, and 0.83, respectively. Moreover, the diagnostic performance was superior for blood-based miRNAs and the use of panels rather than single miRNA [18].

11.3.3 BlCa Serum Protein Biomarkers

A number of serum biomarkers have been associated with disease prognosis and clinicopathologic indices of patients with BlCa. Soluble E-cadherin, uPA, TGFβ1, MMP, IGFBP3, and CYFRA21-1 are associated with BlCa prognosis in terms of cancer-specific mortality, disease progression, and recurrence. Increased serum levels of E-cadherin are associated with aggressive disease stage, grade, and lymph node metastasis (LNM). Similarly, increased circulating levels of uPA in BlCa patients have been associated with prognostic variables such as lymphovascular invasion, LNM, and cancer-specific mortality (CSM). Prognostic correlations in regard to LNM, disease recurrence, and CSM have been provided for elevated plasma TGFβ1, which is overexpressed in BlCa. The elevated MMP levels are associated with LNM and also independently predict CSM. Reduced preoperative levels of IGFBP3 are associated with elevated risk for disease recurrence and CSM in patients with MIBC. High CYFRA21-1 levels are often associated with advanced high-grade tumors [19, 20]. A prognostic role for serum VEGF was revealed in NMIBC patients on the oral retinoid fenretinide. After a 20-year follow up, baseline serum VEGF levels in the top quintile ($\geq$350 pg/ml) were significantly associated with shorter OS and BlCa survival. Serum VEGF levels and smoking status were independent prognostic factors in multivariate analysis [21]. Probably when used as a panel, these circulating proteins may be useful prognostic biomarkers of BlCa.

11.3.4 Circulating BlCa Cells

Circulating BlCa cells (CBlCaCs) have been analyzed in relation to various clinical utility such as diagnosis, prognosis, and staging. Many studies have focused on their prognostic relevance, which reveal their potential as adjuncts to patient management. Although many analyses used the FDA-approved CELLSEARCH® system, molecular approaches and isolation based on size and other techniques have been employed as well.

11.3.4.1 CBlCaC Characterization Using CELLSEARCH® Technology

Naoe et al. used the CELLSEARCH® method and were able to detect CBlCaCs in 57.1 % of patients with metastatic disease but not in patients without metastasis [22]. In another study, the detection rate was 44 % in metastatic urothelial cancer patients, and the number of CBlCaCs positively correlated with the number of metastatic sites, i.e., the numbers were higher in patients with $\geq$2 metastatic sites than those with $\leq$1 site [23]. Expectedly, CBlCaCs were detected at a frequency of 17 % in patients with localized urothelial cancer compared to 50 % in those with metastatic disease. However, the presence of both metastasis and CBlCaCs was strongly associated with poor survival outcome [24]. Similar detection rate of 18 % was demonstrated in patients with NMIBC, but again the presence of circulating cancer cells was associated with shorter time to recurrence and higher T stage [25]. To predict extravesical spread before surgery (staging), CBlCaCs were not a robust biomarker. While the detection rate was lower than the previous studies (21 %), the accuracy in predicting extravesical involvement prior to surgery was only 57.9 % [26].

11.3.4.2 CBlCaC Characterization Using Molecular and Other Methods

Osman et al. used molecular methods targeting uroplakins (*UP*)Ia, Ib, II, and III as well as *EGFR* transcripts to characterize CBlCaCs [27]. The combination of UPIa/UPII had the best accuracy, with a sensitivity of 75 % and specificity of 50 % in CBlCaC detection. In this series, all eight patients who developed disease recurrence on follow-up were positive for *UPIa*/*UPII* mRNA at presentation. Immunomagnetic capture followed by molecular targeting of *CD45*, *KRT8*, and *BIRC5* mRNA was used to characterize CBlCaCs and correlated with DFS. This assay enabled CBlCaC detection at a frequency of 44 % in patients with T1G3 NMIBC, with many (92 %) circulating cells expressing *BIRC5* mRNA. Multivariate analysis revealed the presence of CBlCaCs to be a significant independent predictor of DFS [28]. In search for a diagnostic biomarker of BlCa, Qi et al. used folate receptor-α ligand-targeted PCR to detect CBlCaCs as diagnostic biomarkers [29]. Quantitatively, the levels of folate receptor-α ligand were much higher in patients with transitional cell carcinoma of the bladder compared to healthy controls, and the diagnostic AUROCC was 0.819. A meta-analysis of the diagnostic potential of CBlCaCs reveals a low sensitivity of 35.1 %, but specificity was much higher at 89.4 %. The PLR and NLR were 3.77 and 72 %, respectively. CBlCaC-positivity was associated with advanced stage disease (III–IV) (OR, 5.05). While it may be useful for confirming advanced stage disease, CBlCaCs are not suitable early detection biomarkers [30].

Novel approaches are still being sought to increase the detection sensitivity, especially in patients with localized disease but with micrometastasis. Alva et al.

used IsoFlux, which outperformed the CELLSEARCH® method in CBlCaC detection [31]. Additionally, methods such as separation based on size (MetaCell® device) are enabling the isolation of viable cells for culture and other studies [32, 33].

11.4 Summary

- BlCa is the most commonly diagnosed cancer of the urinary tract, with disproportionately more cases in men than women.
- Tobacco smoke and occupational carcinogen exposure are the main risk factors of BlCa.
- The majority of cancers of the urinary bladder are urothelial carcinomas.
- These urothelial carcinomas fall under two pathologic groups: low-grade superficial and less aggressive non-muscle invasive cancers (NMIBCs) and aggressive high-grade muscle invasive cancers (MIBCs).
- The two groups are characterized by different molecular pathology. Both appear to require an initial 9p/9q deletion. However, NMIBCs often harbor *FGFR3* mutations and are genetically stable, while MIBCs are characterized by *TP53*, *RB*, and *PTEN* mutations and are genetically unstable.
- Biomarkers of BlCa are mostly discovered and validated in urine.
- Circulating BlCa biomarkers will be clinically useful in disease prognosis and treatment decision-making.
- Emerging circulating BlCa biomarkers are ctDNA (e.g., detection of *TP53* mutations and LOH) and miRNAs.
- A number of serum proteins are elevated in patients with advanced stage disease and are associated with disease prognosis.
- Circulating BlCa cells are potential prognostic biomarkers under development.

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Chapter 12
Prostate Cancer Biomarkers in Circulation

Key Topics

- Screening for prostate cancer (PrCa)
- Molecular pathology of PrCa
- Circulating cell-free DNA content as PrCa biomarker
- Circulating PrCa epigenetic biomarkers
- Circulating PrCa noncoding RNA biomarkers
- Circulating PrCa protein and metabolomic biomarkers
- Circulating PrCa cells

Key Points

- PrCa is the most commonly diagnosed male cancer. Increasing screening efforts have led to early detection of curable diseases; however, this has been associated with the criticisms of overdiagnosis and overtreatment. Biomarkers that can forecast the biologic behavior of low-grade tumors are needed.
- Because of the less than optimal accuracy of the PSA test, there have been great interests in uncovering circulating biomarkers for PrCa.
- These research efforts have generated numerous discovery biomarkers needing validation. Some promising ones (e.g., IL-6 and TGFβ1 levels) are incorporated into the Kattan nomogram. Circulating PrCa cells demonstrate prognostic and predictive clinical utility.

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12.1 Introduction

Globally, prostate cancer (PrCa) is the most commonly diagnosed non-cutaneous malignancy, and a major cause of cancer-related deaths in men. In 2012, the estimated incidence was 1.1 million with 307,000 case fatalities worldwide. The 2016 estimates are 180,890 new cases with 26,120 expected deaths for the US. The mortality rate falls significantly below the incidence rate, partly due to intense screening detection of mostly indolent tumors, especially in the more developed world. Consistent with this possibility, most cases of PrCa (~70 %) are diagnosed in the more developed parts of the world such as Australia, New Zealand, North America, and Europe. The geographic regions second to the aforementioned with increased PrCa diagnosis include the Caribbean, South America, and South Africa, with the lowest incidence rates in Asia.

Prostate cancer is screened for by the prostate-specific antigen (PSA) test and digital rectal examination (DRE), whereby an abnormal finding in either of these triggers a biopsy for evident histopathologic diagnosis. But because of biopsy sampling errors (because it randomly samples small volume of the gland), false negative outcomes are concerns to the urologic community. Similarly, clinical trials have shown that the finding and treatment of clinically insignificant tumors are even of a major concern not only from an economic perspective but also the physical and psychological harm it brings to the patient. This heightened evaluation of men over 50 leads to overdiagnosis and overtreatment.

Similar to breast and other cancers, PrCa is very heterogeneous, with unpredictable disease course. Some tumors may grow slowly (indolent cancers) and yet others can be very aggressive and extremely lethal. Due to the obvious limitations of PSA, and that DRE-detected tumors are not early cancers, the need for accurate early detection biomarkers is being actively sort after. Of even much importance are biomarkers that can accurately differentiate between indolent and aggressive cancers (the Holy Grail PrCa biomarker). Thus, companion diagnostic biomarkers that can be assayed noninvasively, preferably at the community level or at home (the lab-on a chip technology), will be an extremely helpful armamentarium.

Castrate-resistant PrCa (CRPC) poses a major challenge in PrCa management. Prostate cancers are addicted to androgen signaling for survival. Androgen deprivation is therefore a mainstay therapeutic strategy; however, the PrCa cell eventually becomes resistant to these treatments, which is a major issue with PrCa management. While advances are being made to develop alternative and more effective pharmacologic agents, docetaxel remains the first-line chemotherapeutic agent. While this treatment has major toxicity issues, only ~50 % of men will demonstrate some response. Noninvasive companion diagnostic and predictive biomarkers are needed, and these should be included in drug development protocols.

12.2 Screening Recommendations for PrCa

In general, there are no consensus guidelines or recommendations for PrCa screening. The reason is simply due to the fact that the PSA test is not accurate enough as a screening biomarker. Though it may have some benefits, many men with elevated PSA are subjected to invasive biopsy procedures, with possible detection of indolent cancers. Often many of these men are also subjected to unjustifiable treatments with their associated complications. The concept of overdiagnosis and overtreatment is well established in the PrCa field. Thus, screening recommendations vary considerably between various agencies and hospitals. The US Preventive Services Task Force, for instance, does not recommend PSA screening for PrCa. Similarly, because many PrCas are indolent, screening for men aged 79 or older is not recommended.

The American Cancer Society, however, has cautious recommendations aimed at making sure the individual and his healthcare team make an informed decision on screening. It is left open for men and their healthcare providers to decide on screening based on risk factors the person may have in relation to the potential benefits, prior to embarking on screening. This decision-making process takes into account the age of the individual, as well as family history of PrCa, and should start at:

- Age 50 for the average-risk individual who has a life expectancy of at least 60 years
- Age 45 for men with a first-degree relative diagnosed with PrCa at an age <65, as well as African-Americans
- At age 40 for men at elevated risk because they have as least two first-degree relatives diagnosed with the disease at an earlier age

The screening program involves periodic PSA testing and DRE. The initial or prior screening results should inform subsequent screening intervals. Men with PSA of <2.5 ng/mL should be screened every couple of years, while those with ≥ 2.5 ng/mL need annual testing. The patients' overall health status, PSA values, and preferences are to be considered in the screening process.

The Memorial Sloan Kettering Cancer Center (MSKCC) has recommended guidelines equally aimed at reducing overdiagnosis and yet able to detect significant disease early. They recommend screening begin at age 45. Men with PSA levels ≥ 3 ng/mL should consider having a biopsy, while those with levels between 1 and 3 ng/mL should have repeat testing every 2–4 years. With the lowest-risk category (PSA <1 ng/mL), decision on further testing should be based on stratified ages; for men aged 45–49, repeat testing should occur at by ages 51–55; those aged 50–59, repeat at age 60; and for those aged 60–70, there should be no further testing. Prior PSA levels and health status of men 71–75 should guide further testing, and for men ≥ 76, testing is not recommended.

12.3 Molecular Pathology of PrCa

With advancing age, the normal prostate glandular epithelium undergoes changes that may lead to PrCa. Inflammatory conditions may cause proliferative atrophic lesions (PIA). Similarly, prostatic intraepithelial neoplastic (PIN) changes may advance to high-grade lesions (HGPIN). Both PIA and HGPIN are precursor lesions of localized PrCa that may metastasize.

Prostate cancer demonstrates a unique molecular pathology, quite different from other solid tumors. Unlike many solid tumors, mutations are rare events, while epigenetic alterations play a major role in PrCa development and progression. Similarly, genomic rearrangements leading to fusion gene formation underlie PrCa development and progression. Genome-wide interrogation indicates PrCa cells harbor 1–2 mutations per megabase, and the frequency appears to increase with disease progression. These global genomic profiles enabled identification of a novel gene (*SPOP*) that is commonly mutated in PrCa, especially those lacking*TMPRSS2-ERG* fusions that characterize 27–79 % of PrCas and some precursor lesions.

12.3.1 Epigenetic Alterations in PrCa

The epigenome is altered in PrCa, and this is often an early event in disease progression. DNA methylation changes, histone modifications, and altered miRNA expression have all been demonstrated features of PrCa.

12.3.1.1 DNA Methylation in PrCa

Well established in the molecular progression model of PrCa is early gene promoter hypermethylation. Currently, there are over 50 genes silenced through promoter hypermethylation in PrCa. In many instances, these genes harbor infrequent mutations, LOH, deletions, or other genetic aberrations, except the epigenetic changes, which are often the primary mechanism of their silencing. Of interest, several of the hypermethylated genes in PrCa are age associated, being equally hypermethylated in association with the aging process. This offers further evidence for age being a risk factor of PrCa. Some of the commonly hypermethylated genes are *GSTP1*, *APC*, *RASSF1A*, *RARβ2*, *CCND2*, *PTGS*, *ESR1/2*, *CDKN2A*, *EDNRB*, *HIC*, *MDR1*, *CAV1*, *CDH1*, and *BCL2*. Less well studied is gene activation via promoter hypomethylation. However, global hypomethylation is associated with metastatic PrCa (mPrCa), and ~50 % of PrCas harbors hypomethylation, especially at DNA repeats such as LINE sequences. Several overexpressed genes due to promoter hypomethylation are also noted, and these include *S100P*, *PLAU*, *WNT5A*, *CAGE*, *HPSE*, *CRIP1*, and *CYP1B1*.

12.3.1.2 Histone Modifications in PrCa

Posttranslational modifications of histone proteins in association with chromatin remodeling underlie altered gene expression in PrCa. Noteworthy are altered expression of genes such as *EZH2*, *HDAC1*, and lysine-specific demethylase 1 (*LSD1*) involved in histone modification. *EZH2*, a histone methyltransferase involved in trimethylation of histone H3K27 (and also dimethylation of H3K9), is highly overexpressed in PrCa. Additionally, *EZH2* overexpression is associated with silencing via promoter hypermethylation of PrCa-associated genes such as *NKX3.1* and *DAB2IP* involved in EMT. *HDAC1* upregulation is also a feature of PrCa, and this is more often associated with castration-resistant prostate cancer (CRPC).

12.3.1.3 MiRNA Alterations in PrCa

The new players in epigenetic regulation are miRNAs. Several miRNAs are deregulated in PrCa with yet to be fully deciphered functional importance. Some mechanisms of acquisition of androgen independence and hence CRPC emergence are mediated by miRNAs. Loss of miR-125b is associated with androgen independence. Similarly, reduced expression of miR-488 increases expression and hence transcriptional activity of androgen receptor (AR). Additionally, androgen independence is under the control of miR-221/222, and miR-616, all of which are deregulated in PrCa. MiR-331-3p, downregulated in PrCa, controls *HER2* expression and AR signaling. Decreased miR-146a is associated with hormone-refractory PrCa. Additionally, several miRNAs are deregulated in PrCa, and these target important genes in PrCa cells. MiR34c targets and destroys *BCL2* and *E2F3* and hence is downregulated in PrCa, and miR-34a also downregulated in PrCa targets *SIRT1* and *BCL2*. MiR-21, upregulated in PrCa, targets *PTEN* and *PDCD4*, while downregulation of miR-201 enhances EMT. Several hundreds of other miRNAs including let -7c, miR-15a, miR-16-1, miR-125, and miR-145 with reduced expression in PrCa target *RAS*, *E2F3*, *BCL2*, and *MCL1*. Thus oncomirs (upregulated) and tumor suppressormirs (downregulated) characterize prostate carcinogenesis.

12.3.2 Genetic Alterations in PrCa

Chromosomal abnormalities associated with aberrant oncogene and tumor suppressor gene expressions, as well as deregulated polycomb and developmental regulatory genes, modulate prostate carcinogenesis. Efforts at uncovering these genetic changes have led to the development of valid biomarkers for PrCa.

12.3.2.1 Chromosomal Aberrations in PrCa

Standard cytogenetics has not been that good for analysis of epithelial tumors because of the need for fresh samples, and also epithelial cancer cells grow poorly in vitro. Cytogenetic approaches have thus been very useful in analysis of hematologic malignancies. The evolution of comparative genomic hybridization (CGH) technology has enabled structural genomic aberrations to be scored in several epithelial cancers. The principle of CGH is simply a competition between normal and tumor DNA, each labeled with a distinct fluorochrome for hybridization on normal DNA template. This normal DNA template could be metaphase chromosomes of a healthy person (cCGH) or known genomic fragments immobilized on a microarray surface (aCGH). Fluorescence ratios measured after hybridization enable detection of chromosomal loses and gains (genomic imbalances).

A large number of PrCas (up to 90 %) demonstrate some aspects of DNA copy number imbalances. Importantly, most cancers with copy number abnormalities are advanced pathologic or clinical stage disease. Over half of organ-confined PrCas do not harbor any such lesions, but as many as 89 % of metastatic cancers have copy number changes. Thus progressive acquisition of genomic imbalances propels PrCa development.

Chromosomal losses at 8p (in particular) and 6q, 10q, 13q, 16q, and 18q are very common. Less commonly involved are chromosomal gains at 7q and Xq. But 8q gains are common. Chromosome 8q gain and 13q loss are markers of metastatic extracapsular disease, independent of Gleason score. 8q gain is an independent prognostic predictor of poor disease-specific survival. Array CGH offers better resolution because it detects 2.7–3.4-fold changes. This method has been used to show that losses at 8p23 and gains at 11q13 are associated with advanced metastatic disease. 8p loss is present in ~30 % of organ-confined and ~50 % of metastatic cancers. A very large region spanning ~12 Mbp (8p21.2–8p22) has been implicated. This region contains over 50 genes, but the prostate- and testis-specific androgen regulated homeobox gene, *NKX3.1* at 8p21, has been well studied. The loss of this region occurs very early in PrCa, and its effects on prostate carcinogenesis appear to involve the PTEN/AKT pathway. Haploinsufficiency of *NKX3.1* is insufficient to trigger cancer (not a typical TSG). Inactivation causes PIN lesions, and overexpression reduces PrCa growth. Other genes of interest in this region are the TNFR family members including *TNFRSF10*, *TNFRSF10a,* and *TNFRSF10b*. These genes may act synergistically in PrCa development.

12.3.2.2 Oncogenes and Tumor Suppressor Genes in PrCa

There are several genes that are overexpressed in PrCa, but the two well-established oncogenes are *MYC* and the *TMPRSS2-ETS*. The *MYC* locus on chromosome 8q24 is often amplified in PrCa, and ~30 % of PrCas harbor *MYC* amplifications leading to overexpression. MYC is capable of transforming prostatic epithelial cells.

Additionally, MYC levels are higher in metastatic PrCa (mPrCa) than localized PrCa (lPrCa), and the elevated levels in advanced stage disease are associated with worse prognosis.

While uncommon in solid tumors, fusion genes appear to play an important role in PrCa pathology. Gene fusion between the 5′ untranslated regions of androgen-regulated transmembrane protease serine 2 (*TMPRSS*) to the 3′ exon of erythroblast transformation-specific (*ETS*) transcription factor family members is common in PrCa. *TMPRSS* and *ERG* (a member of the ETS transcription factor family of genes) map to chromosome 21q22, and the two genes are separated by less than 3 megabases. In a large percentage of PrCas (30–80 %), and some PIN lesions (up to 21 %), intergenic deletion of segments between these two genes leads to the *TMPRSS2-ERG* fusion rearrangements, of which the commonest is *TMPRSS2-ETS* fusion. The remaining fusion genes in PrCa are accounted for by other rearrangements of these genes. These fusion genes have several roles in PrCa including conferring androgen independence and are often associated with worse prognosis. Their noninvasive diagnostic and prognostic use in PrCa is highly pursued using primarily urinary and prostatic fluid samples. Other fusion genes demonstrated in PrCa samples include *RAF1-ESRP1*, *ESRP1-RAF1*, *C15orf21-MYC,* and *SLC45A3-BRAF*. Their roles in PrCa are being elucidated.

Similarly, alterations in TSGs are few in PrCa, but two well established are *PTEN* and *RB*. PTEN is a major negative regulator of the PI3K/AKT pathway. Consequently, *PTEN* loss through LOH, methylation, and mutations is observed in a vast majority (~70 %) of PrCas. Loss of a single allele is an early event in PrCa, and this may play a role in PrCa initiation through PI3K/AKT signal inhibition of *NKX3.1* expression. Homozygous loss of *PTEN* is a feature of mPrCa. PTEN can act in concert with other altered genes such as elevated expression of *HER2/3*, *BMI1*, and loss of *TP53* to promote prostate carcinogenesis. The *RB* tumor suppressor is also lost in a vast majority (~70 %) of CRPC. This leads to E2F1 translocation into the nucleus and the induction of transcription of multiple genes involved in cell cycle progression as well as the *AR*.

12.3.2.3 EZH2 and BMI1 Alterations in PrCa

Polycomb repressor complexes 1 and 2 (PRC1 and 2) are involved in gene silencing and are upregulated in many cancers. PRC1 enzymatically modifies histones and chromatin structure leading to gene silencing. *BMI1* is a ring domain-containing enzyme that participates in PRC1 functions. PRC2, however, represses gene expression through histone methylation on lysine 79 of histone H3 (H3K79me2/3), and this is mediated through its component, EZH2. Both BMI1 and EZH2 are deregulated in PrCa. EZH2 for instance is under the control of MYC, TMPRSS2-ERG, and miRNAs and is also upregulated through gene amplification. *PTEN* loss of function and activated PI3K/AKT signaling can phosphorylate BMI1, and this interaction is involved in PrCa progression to invasive cancers. BMI1 activity is also enhanced by EZH2.

12.3.3 Developmental Gene Alterations in PrCa

Prostate glandular formation and cellular diversification are regulated by *NKX3.1*, *AR*, and *FOXA1*. Abnormal expression or reactivation of these genes can cause prostatic glandular hyperplasia leading to the development of neoplastic lesions and PrCa.

NKX3.1 is a homeobox developmental gene located on chromosome 8p21, a chromosomal region with LOH in ~50 % of lPrCa and up to 80 % of mPrCa. It is a transcriptional repressor expressed early in prostate glandular development and differentiation and is required for all stages of its development. Loss of *NKX3.1* carries a risk for PrCa development; however, it fails to display typical tumor suppressor functions, because some aggressive PrCas maintain normal expression, and the loss of *NKX3.1* is insufficient to initiate cancer formation. There appears to be interplay between NKX3.1, MYC, and PTEN in PrCa progression. NKX3.1 represses MYC target genes. Thus MYC overexpression and loss of NKX3.1 are associated with the development of PIN. Loss of NKX3.1 also appears to coincide with loss of PTEN, suggesting possible synergistic interactions.

The AR reactivation is a major cause of the emergence of CRPC, following androgen deprivation therapy (ADT). Various mechanisms, including mutations, amplification, splice variants, posttranslational modifications, and synthesis of androgen by cancer cells, are involved in AR activation. In men with lPrCa, treatment with ADT causes gene amplification and overexpression leading to CRPC. Gain of function mutations is also detected quite often in CRPC but not in early stage untreated disease. The most common mutation (T877A) resides in the ligand-binding domain and serves to increase androgen sensitivity. Up to 25 % of CRPC harbors *AR* mutations. Posttranscriptional splicing and/or gene rearrangement generates constitutively active AR splice variants. The most common splice variant (AR3/AV-7) is a 35-kb intragenic tandem duplication of the third exon. Another variant is the Arv567es that results from deletions of exons 5, 6, and 7. These alternative spliced forms are overexpressed in CRPC and possibly induce alternative gene expression signature from those by the wild-type AR. Additionally, posttranslational modifications (PTM) occur in the AR that enhances its functions. These changes are more often associated with CRPC compared to treatment naïve tumors. The most common PTM is phosphorylation of different tyrosine, threonine, and serine residues on the AR. For example, pSer210 can activate the AR in conditions of low androgen levels, and this modification is associated with progression to CRPC.

FOXA1 enhances AR binding to chromatin to induce target gene expression. Expectedly, *FOXA1* is overexpressed in CRPC, and this is associated with poor prognosis. FOXA1-mediated control of AR interaction with chromatin leads to expression of defined gene sets in lPrCa and CRPC. Mutations in the DNA-binding domain of *FOXA1* are uncovered in PrCa, but their functional role awaits elucidation.

An intriguing finding in the development of AR is the presence and overexpression of all the necessary ingredients required for de novo androgen

synthesis by CRPC cells, which suggests that these cells can synthesize and respond in an autocrine fashion to androgens, even in conditions of absence or low circulating androgens, as in ADT.

12.4 Circulating PrCa Biomarkers

The need to complement or replace the PSA test has led to the discovery of several circulating biomarkers for PrCa. Extensively investigated are alterations in ccfDNA, epigenome, noncoding RNA, and proteins. Established as prognostic biomarkers are circulating PrCa cells.

12.4.1 Circulating Cell-Free Nucleic Acid Content as PrCa Biomarkers

The levels of ccfDNA have been quantified in blood from PrCa patients using mainly the PCR technique; however, a few studies have used fluorometric, spectrometric, and dipstick (Invitrogen) approaches. In spite of the inherent technical variables of biomarker development, the available data suggest a potential role for ccfDNA content in PrCa diagnosis and prognosis, especially in men with high-risk indices such as rising PSA and abnormal DRE (Table 12.1).

The pioneering work by Jung and colleagues failed to reveal differences in ccfDNA content between patients with localized PrCa (lPrCa) and controls, although levels were increased in BPH and metastatic PrCa (mPrCa) [1]. However, there was a significant difference in the levels between lPrCa and mPrCa patients, and this was associated with disease prognosis. Subsequent studies by other investigators revealed clear differences in the circulating levels of DNA in cancer patients and controls, achieving a range of sensitivities, specificities, and AUROCC of between 58–88 %, 64–94 %, and 0.708–0.881, respectively. The available data indicate ccfDNA levels could be threefold higher in PrCa patients than controls. The work by Ellinger and colleagues in 2008 is noteworthy [2]. Using samples from 168 patients with PrCa, 42 with BPH, and 11 healthy controls, a sensitivity of 88 %,

Table 12.1 Diagnostic and prognostic potential of ccfDNA content in PrCa

Utility	Diagnostic performance and prognostic utility
Diagnosis	Sensitivity: 58–88 % Specificity: 64–94 % AUROCC: 0.708–0.881
Prognosis	Levels are much higher in metastatic PrCa men than those with localized disease Levels correlate with Gleason score and pT stage Levels are predictive of PSA recurrence and cancer-specific survival Levels predict surgical margin status and extra-prostatic spread

specificity of 64 %, and AUROCC of 0.824 were achieved, proving the potential diagnostic utility of ccfDNA in high-risk men. Papadopoulou et al. had observed elevated ccfDNA with diagnostic potential as well [3]. The cost-effective dipstick method apparently achieved comparable results to the other expensive and labor-intensive methods. The smaller samples investigated by Altimari at al. (64 cancer patients and 45 men with BPH) had a higher performance (sensitivity of 80 %, specificity of 82 %, and diagnostic AUROCC of 0.881) [4]. The multi-biomarker model approach by Chun et al. [5] may be best for enhancing the use of ccfDNA in PrCa detection. Incorporating ccfDNA in a diagnostic model with total PSA and free/total PSA improved PrCa detection accuracy by 5.6 %. While many of these works indicate promise, Boddy et al. failed to reproduce them [6]. Despite this, the verdict is in favor of the diagnostic potential of ccfDNA, if validated and probably incorporated into an algorithm with other useful PrCa biomarkers.

The prognostic potential of ccfDNA levels in men with PrCa has also been shown. Plasma DNA content is in general higher in patients with mPrCa than those with lPrCa. Additionally, these levels are demonstrated to correlate with pathologic stage and significantly predict PSA recurrence after surgery, as well as PrCa-specific survival in patients with metastatic disease.

12.4.1.1 Circulating Mitochondrial DNA Content as PrCa Biomarker

In men with PrCa, the role of ccf-mtDNA has been addressed. MtDNA content was increased threefold in men with mPrCa compared to controls [7]. Noted was the lack of correlation between the content of mtDNA and nuclear DNA. Furthermore, this study demonstrated an association between high ccf-mtDNA content and PrCa-specific survival. The work by Ellinger et al. found that while mtDNA content did not differ between men with lPrCa and BPH, it was an independent predictor of PSA recurrence after surgery [8].

12.4.1.2 DNA Integrity Index as PrCa Biomarker

The clinical importance of DNA integrity index (DII), a measure of necrotic vs. apoptotic DNA in circulation, has been examined in men with PrCa. The measurement of ten different DNA of fragment sizes between 200 and 10 kb in plasma from PrCa patients and healthy controls with DII defined as ratio of long to short fragments revealed higher index in patients than controls. DII achieved a diagnostic sensitivity and specificity of 70 % and 68 %, respectively, with the AUROCC of 0.788. Ellinger et al. amplified <200 bp of *PTGS2* (apoptotic fragments) and >250 of *REPRIMO* (non-apoptotic fragments) and defined apoptotic index (AI) as the ratio of *PTGS2* to REPRIMO DNA fragments [9]. Ironically, there were more short DNA fragments (*PTGS2*) in cancer patients than in men with BPH (suggesting more apoptosis in cancer cells than BPH, but the authors refer to this as

being of noncancerous source), achieving a sensitivity and specificity of 88 % and 64 %, respectively, as a diagnostic. The AI was also significantly higher in cancer patients than BPH and healthy men, achieving a sensitivity of 70 % at and enhanced specificity of 82 %. Short ccfDNA fragments could also predict PSA recurrence after surgery. Feng et al. obtained plasma samples from 96 cancer patients and 122 men with BPH for measurement of ccfDNA levels and DII analysis [10]. DII was defined as the ratio of ALU247bp to ALU115bp fragments. Both of these indices (ccfDNA and DII) could differentiate men with PrCa from controls, which were men with BPH and elevated PSA ($\geq$ 4 ng/ml) but without cancer. Mean plasma ccfDNA content was 19.74 ± 4.43 in PrCa patients compared to 7.36 ± 1.58 in men with BPH ($p < 0.0001$), and mean DII was 0.34 ± 0.05 in cancer patients compared with 0.19 ± 0.03 in the BPH cohort ($p < 0.0001$). The diagnostic performance based on AUROCC was 0.864 for ccfDNA levels and 0.910 for DII. In addition to ccfDNA content, DII also demonstrates clinical potential in PrCa patients.

12.4.2 Circulating PrCa Epigenetic Biomarkers

Prostate cancer is characterized by several gene promoter hypermethylation. However, the most commonly studied somatic gene alteration in PrCa is *GSTP1* promoter hypermethylation that occurs in up to 90 % of PrCas. Similarly, although there are numerous methylated genes in PrCa, *GSTP1* is most extensively studied in body fluids. Available studies have detected *GSTP1* methylation at various frequencies in circulation of PrCa patients. Multiple body fluid samples (serum, plasma, urine, nucleated blood cells, ejaculates) from PrCa and BPH patients were analyzed for *GSTP1* promoter methylation in csb-DNA and ccfDNA templates. Methylation was detected in 90 % of tumors, 72 % of plasma/serum samples, 76 % urine samples, and 50 % of ejaculates. Methylation as a marker of CTCs were detected by MSP in 30 % of PrCa patient samples [11]. In another cohort, tissue and matched preoperative plasma and urine samples from early PrCa and BPH patients were tested for *GSTP1* methylation. Methylation was detectable in 91.3 % of PrCa and 29 % of BPH tissue samples and was detected in up to 53.6 % of plasma and urine samples [12]. *GSTP1* promoter hypermethylation in PrCa and BPH patient plasma samples was explored in another series, and methylation was in 30.6 % of PrCa and matched tissue samples. The majority (92.6 %) of BPH samples were negative for *GSTP1* methylation [13].

12.4.2.1 Circulating PrCa Diagnostic Epigenetic Biomarkers

Multiple studies of gene methylation in circulation using multivariate analytical approaches offer evidence of their clinical potential in PrCa. In an attempt to uncover diagnostic circulating epigenetic biomarkers for PrCa, a multigene CpG island hypermethylation in sera from PrCa patients was conducted. Sera from

226 consecutive patients comprised of 168 PrCa, 42 BPH, 11 healthy controls, and 5 incidental PrCas were subjected to *GSTP1*, *PTGS2*/*Cox2*, *REPRIMO*/*RPRM*, and *TIG1* methylation analysis. *GSTP1* and *TIG1* hypermethylation significantly differentiated PrCa patients from BPH and healthy controls. *GSTP1* methylation in sera, in combination with any of the other genes, was very specific for PrCa (92 %), but sensitivity was low (42–47 %). This assay could help identify men with PrCa despite negative biopsy because of the high specificity [2]. A cohort of samples from a previous study of *GSTP1* methylation as a measure of tumor-derived ccfDNA [14] were examined for apoptotic index, defined as a ratio of the levels of *PTGS2* (<200 bp apoptotic fragments) to those of *REPRIMO* (>250 bp non-apoptotic fragments). The apoptotic index was significantly higher in PrCa than BPH patients and healthy controls. Only a small percentage (1.92 %) of ccfDNA was positive for *GSTP1* methylation. *PTGS2* DNA concentration performed at a sensitivity of 88 % and specificity of 64 % in PrCa detection. The apoptotic index achieved diagnostic sensitivity of 70 %, and specificity of 82 %, and correlated with histologic tumor grade. Kaplan–Meier analysis showed a significant correlation between *PTGS2* fragments or apoptotic index and PSA recurrence after surgery [9].

Analysis of multiple biomarkers for PrCa detection was conducted by targeting methylation of *GSTP1*, *RARβ2*, and *RASSF1A* and allelic imbalance (AI) at six polymorphic loci in serum samples from men with stage I–IV PrCa and healthy controls. Forty-seven percent of PrCa patients had one or more AI. Methylation was detected in 28 % of serum samples. The use of both markers increased PrCa detection to 63 %. Importantly, 63 % of PrCa patients with normal PSA were detected by this assay, and the sensitivity was 89 % [15]. Another study attempting to prove the value of biomarker panel analysis in body fluids (serum, urine) for PrCa screening targeted circulating levels of PSA, AMACR, and MMP-2, as well as methylation of *RASSF1A* and *GSTP1*. Serum and urine from 113 men with lower urinary tract symptoms were obtained for analysis. With an AUROCC of 0.706 indicated that serum measurement of MMP-2 is superior to both PSA and AMACR. The methylation status of both genes was not much better than biomarker combination, which increased the overall AUROCC to 0.788 with a sensitivity of 57.1 % and specificity of 96.6 % [16]. *RARβ2* methylation in serum of PrCa and BPH patients as a novel biomarker of PrCa revealed a high detection rate of 92.9 % in samples from PrCa patients and in 10.7 % BPH patient sera, indicative of needed further exploration of this biomarker [17].

Meta-analysis provides objective information for evidence-based medicine. Thus, a meta-analysis of *GSTP1* methylation in sera, plasma, whole blood, urine, ejaculate, prostatic secretions has been conducted. This work included 22 studies that met selection criteria. The pooled plasma, serum, and urine data yielded a sensitivity of 52 %, but the specificity was as high as 89 %. There was high specificity with regard to sample type, methods, and primer sequences and locations. While the sensitivity is similar to that of PSA, the much higher specificity than PSA suggests it can be used as a complementary assay to PSA to help reduce

the false-positive rates of PSA and importantly in patients with biopsy sampling errors [18].

12.4.2.2 Circulating PrCa Epigenetic Biomarkers and Clinicopathologic Variables

Analyses of circulating *GSTP1* methylation have also been correlated with various clinicopathologic features of PrCa patients. Preoperative sera from men with PrCa treated with radical prostatectomy and those with negative biopsies were analyzed for *GSTP1* methylation. Additional set of patients comprised of lPrCa patients treated with prostatectomy, those with PSA recurrence within 2-year median follow-up, and those free of disease after 3-year median follow-up were further tested. *GSTP1* methylation was in 12 % of lPrCa, 28 % of metastatic PrCa patients, as well as in 15 % of those with recurrences, but in none of those free of disease. Multivariate analysis indicated that *GSTP1* methylation was a significant predictor of PSA recurrence with a HR of 4.4 [19]. In another study, serum samples from patients with hormone-refractory PrCa (HRPC), early stage disease, and healthy controls were subjected to methylation analysis of *GSTP1*, *AR*, and *SFN* methylation statuses. Methylation frequencies in sera from HRPC patients were 32.2 % for *GSTP1*, 40.3 % for *AR*, and 86.6 % for *SFN*. People with early stage disease as well as healthy controls also showed variable levels of methylation in sera. The methylation of *GSTP1* and *SFN* significantly differentiated HRPC patients from healthy controls, and *GSTP1* hypermethylation in sera from HRPC patients significantly correlated with metastasis and cancer differentiation status [20].

Earlier studies have shown that *GSTP1* promoter hypermethylation in preoperative serum samples from patients with lPrCa is predictive of early PSA failure after surgery. Thus, sera from 192 men with lPrCa, 18 with metastatic HRPC, and 35 with biopsy negative for PrCa were analyzed targeting CpG island hypermethylation of eight genes (*MDR1*, *EDNRB*, *CD44*, *NEP*, *PTGS2*, *RASSF1A*, *RARB*, and *ESR*). Of all the genes, only *MDR1* was positive in 38.2 % of lPrCa patients and in 16.1 % of those with recurrent disease after surgery. The remaining seven genes were negative in sera from patients with lPrCa. All the genes except *CD44*, *PTGS2*, and *ESR* were methylated at different frequencies in sera form patients with mPrCa, with the most frequently methylated gene being *MDR1* (83.3 %). Thus, a panel of well-selected genes in serum could define metastatic HRPC [14].

12.4.3 Circulating PrCa Genetic Biomarkers

The chromosomal alterations in PrCa are examined in circulation as well. LOH in plasma as a tool for PrCa screening has been explored using a panel of 15 polymorphic markers of known TSGs. DNA concentration was higher in cancer than BPH

patients. LOH was more frequent in samples from PrCa (34 %) than in BPH (22 %) patients. In this cohort, LOH was highest (22 % each) at 3p24 (*THRB* locus) and 8p21 (D8S360) in PrCa samples, and these differed from BPH loci with frequency of 6 % at each of D8S286, D8S360, D9S1748, and D11S898 [21]. Schwarzenbach's group further used 15 markers for LOH analysis of tissue, plasma, and bone marrow aspirates from PrCa patients. LOH was as high as 72 % in tissue samples but 56 % and 44 % in bone marrow and plasma samples, respectively. Different markers were positive in different samples (evidence of tumor heterogeneity). For example, the highest blood markers were D8S360/D10S1765, and the highest bone marrow markers were THRB/D8S137. Twenty-two percent of patients with no clinical evidence of metastasis had tumor cells in bone marrow, of which 16 % had LOH detectable in bone marrow plasma. This is the first observation of the presence of tumor DNA in blood and bone marrow plasma of PrCa patients with implications for early detection of micrometastasis [22]. Another study by this group involved the use of 13 polymorphic markers on samples from a large patient population to establish the frequencies of AI in PrCa patients in relation to clinical and pathologic variables. Allelic imbalance was detected at a frequency of 11 % in plasma and 34 % in tissue samples. LOH at D7S522 in plasma was associated with increased prostate volume, and LOH at *THRB* with PSA and percent-free PSA [23]. With 12 polymorphic markers used to examine plasma from patients with lPrCa and mPrCa, the frequency of LOH was 10 % and 12 % in localized and metastatic cancer samples, respectively. However, fractionation of plasma DNA enabled increased detection of LOH in low molecular weight fractions (23 %) compared to high molecular weight fractions (7 %). Most frequent site of LOH was at marker D11S898, and marker combinations D6S1631/D8S286/D9S171 and D8S286/D9S171 were associated with increasing Gleason scores [24]. In an attempt to develop a multi-biomarker serum assay for PrCa early detection, six polymorphic markers on six chromosomes, in addition to promoter methylation of *RASSF1*, *RARβ2*, and *GSTP1*, were examined. Allelic imbalance and methylation were detected in 47 % and 28 %, respectively. Of noteworthy, when the two classes of biomarkers (LOH and methylation) were combined, the detection of PrCa in men with apparently normal PSA was increased to 63 %. This assay achieved a sensitivity of 89 % for PrCa detection [15]. Microsatellite alterations that are frequent in PrCa can be detected and measured in circulation of cancer patients, and this has potential clinical implications worthy of exploration.

12.4.4 Circulating PrCa Coding RNA Biomarkers

The diagnostic utility of circulating transcripts, mostly targeting *hTERT* mRNA, has been assessed by a number of studies. Plasma from men with elevated PSA and healthy controls were assayed for *hTERT* mRNA. Median *hTERT* mRNA values were significantly much higher in cancer than control samples. Importantly, patients with prostatitis had lower levels than the PrCa cohort. At a defined cutoff,

hTERT mRNA achieved a sensitivity of 81 % and specificity of 60 % for PrCa detection. Its utility in conjunction with PSA to improve PrCa detection has been suggested [25]. Plasma *hTERT* mRNA as a biomarker for differentiating localized from locally advanced PrCas, as well as its prognostic utility, was evaluated using univariate and multivariate analysis in comparison with conventional tumor markers. Plasma *hTERT* mRNA and serum PSA levels were significantly elevated in samples from locally advanced PrCa patients than men with localized disease. Compared to PSA, plasma *hTERT* mRNA was less sensitive (83 % vs. 100 %) but highly specific and accurate with a higher positive likelihood ratio for differentiating localized disease from locally advanced disease. Multivariate analysis revealed *hTERT* mRNA and age (but not PSA) could predict advanced disease. For prognostic utility, plasma *hTERT* mRNA and serum PSA levels could predict biochemical recurrence in univariate analysis. Multivariate analysis identified Gleason score and PSA as significant predictors of biochemical recurrence. Although *hTERT* mRNA levels showed a positive prognostic trend, it failed to reach significance [26]. A follow-up study by this group examined both the diagnostic and predictive use of plasma *hTERT* mRNA in PrCas. The study design recapitulates the previous one. Plasma from patients with elevated PSA ($n = 105$) and healthy controls ($n = 68$) was assayed. Plasma *hTERT* mRNA was equally sensitive (85 % vs. 83 %) but more specific (90 % vs. 47 %) than PSA. Plasma *hTERT* transcript levels were significantly associated with poor prognosis and were an independent predictor of PrCa. Univariate analysis showed plasma *hTERT* mRNA (again and not PSA) could predict biochemical recurrence. Multivariate test identified *hTERT* and tumor stage as significant determinants of biochemical recurrence [26].

12.4.5 Circulating PrCa Noncoding RNA Biomarkers

Both short (miRNA) and long noncoding RNAs have been investigated as circulating PrCa biomarkers. Circulating miRNAs have been extensively explored for PrCa diagnosis, prognosis, therapy predictions, and monitoring for recurrence.

12.4.5.1 PrCa MiRNA Biomarkers

Several miRNAs show deregulated expression in PrCa and appear to play important roles in the molecular pathology of this disease. They control important signaling pathways involved in PrCa progression, metastasis, and treatment resistance. Upregulated are oncomirs, including miR-21, miR-375, and miR-186/96/182 and miR-221–222 clusters, while downregulated are tumor suppressormirs such as miR-200 family, miR-143/145 cluster, and miR-205. Additionally, profiling studies have uncovered a plethora of deregulated miRNAs in PrCa.

Functionally, miR-21, for example, enhances tumor progression, invasion, and metastasis through inactivation of tumor suppressor genes such as *PTEN*, *RECK*,

PDCD4, and *TPM1*. MiR-221/miR-222 cluster is markedly elevated in mCRPC and is involved in cell cycle control as well as EMT. Similarly, decreased tumor suppressormir levels promote PrCa progression through loss of control on established oncogenes such as *KRAS* and *BCL2*. As an example of their functional relevance, miR-205 that is downregulated in mPrCa targets *BCL2*, *PKCε*, and several MAPK/ERK pathway genes, while miR-143 targets include *KRAS*, *BCL1*, *ERK5*, *ELK1*, and *MYO6*. Some of these miRNAs including miR-21, miR-141, miR-200a, miR-200b, miR-200c, and miR-375 show differential circulating levels between men with PrCa and healthy controls.

Circulating PrCa Diagnostic MiRNA Biomarkers
Several technologies have been applied to identify circulating miRNAs that will differentiate early stage PrCa from BPH and healthy control men. A number of miRNAs including let-7c, let-7e, miR-21, miR-30c, miR-106a, miR-107, miR-221, miR-622, miR-1274, and miR-1285 have shown potential as diagnostic biomarkers of PrCa with some demonstrated remarkable accuracies with AUROCC of up to 0.924.

Mahn et al. explored the diagnostic potential of oncogenic miRNAs (let-7c, miR-26a, and miR-195) and found that the levels were indeed much higher in the peripheral circulation of cancer patients than men with BPH and normal healthy controls [27]. While ten circulating miRNAs could differentiate between men with metastatic and localized cancer from healthy men, the best diagnostic model was provided by a panel of miR-106a and miR-1274 that achieved a remarkable AUROCC of 0.928 [28]. When a number of known PrCa miRNAs were examined in plasma from men with both lPrCa and mPrCa, miR-21 and 221 were much elevated in cancer patients. Kotb et al. then demonstrated the diagnostic potential of circulating miR-21 and miR-221 [29]. These miRNAs were assayed in men scheduled for TRUS-guided biopsy for biochemical and/or clinical suspicion of cancer. MiR-21 and miR-221 were elevated in 90 % and 80 %, respectively, in the men with cancer. Diagnostic performance indicated miR-21 was superior, with both sensitivity and specificity of 90 % in differentiating men with PrCa from those without cancer. Bryant et al. observed deferential expression of 12 miRNAs in PrCa patients vs. controls and found the greatest fold change to be miRNA-107, but this was not superior to PSA as a diagnostic biomarker (AUROCC 0.62 vs. 0.79 for PSA) [30]. Additionally, Chen et al. uncovered a promising panel of five plasma miRNAs (let-7c, let-7e, miR-30c, miR-622, and miR-1285) using Illumina microarray platform, which could differentiate cancer patients from BPH and healthy controls with AUROCC of 0.924 and 0.860, respectively [31].

Circulating PrCa Prognostic MiRNA Biomarkers
Prognostic biomarkers are very important in PrCa, especially if they can be used to stratify which early stage cancers will be indolent from those with propensities to metastasize (biologic behavioral predictions of early cancer). Also of importance will be biomarkers that can identify cancers likely to recur after treatment. The promise of miR-141, miR-298, miR-346, and miR-375 that are elevated in sera from patients with castrate-resistant metastatic disease is worth exploring.

Circulating miR-141 alone predicts clinical outcome and correlates with PSA changes. Tumor expression of miR-375 is negatively associated with biochemical recurrence after surgery. Serum miR-141 and miR-375 levels are associated with established prognostic indices such as tumor stage, Gleason scores, and lymph node metastasis, suggesting their potential use as prognostic biomarkers. Serum miR-195 and let-7c significantly correlate with Gleason scores as well.

The miR-141 Story in PrCa

Prostate cancer-circulating miRNAs with prognostic potential include miR-141, miR-200b, and miR-375. Circulating levels correlate with Gleason scores and lymph node involvement. Indeed, their increasing levels mirror tumor progression form low-risk to high-risk and eventually metastatic disease individuals [30, 32]. In other studies, circulating miR-21 and miR-141 levels have been correlated with PrCa risk indices such as CTC counts, PSA, and LDH levels, while miR-20a, miR-21, miR-145, and miR-221 levels correlated with D'Amico and cancer of the prostate risk assessment (CAPRA) scores.

Mitchel's seminal proof of concept that tumor-derived miRNAs could be detected in the circulation was conducted on mouse PrCa xenographs. With regard to humans, a few prostate-specific miRNAs were investigated, and serum levels of miR-141 were much higher in mPrCa patients compared to controls [33]. MiR-141 is involved in EMT and hence ties in well with elevated expression in mPrCa. Also using the TRAMP mouse model, Selth et al. identified four circulating miRNA (miR-141, miR-298, miR-346, and miR-375), to be differentially expressed between mice with advanced stage disease and healthy controls [34]. These same circulating murine miRNAs were elevated in sera from patients with metastatic CRPC. MiR-375 levels inversely correlated with biochemical failure. Subsequent analysis of circulating miRNAs in men with lPrCa and mPrCa confirmed and validated the association of miR-141 and miR-375 with advanced stage disease, systemic disease, as well as other clinicopathologic features such as tumor stage and Gleason scores in men with lPrCa prior to surgery [30, 32, 35]. In a multicenter prospective evaluation of serum miR-26a-1 and miR-141, it was demonstrated that while the levels of both miRNAs were comparable between biopsy positive and negative patients, miR-141 was significantly increased in patients with higher Gleason scores [36]. Circulating miR-141 and miR-221 levels were much elevated in men with mPrCa, and miR-141 was associated with clinical tumor progression and PSA levels and could differentiate mPrCa patients from healthy controls [33, 37]. Moreover, tissue levels of miR-221 were predictive biomarker of PrCa-related death [38]. Consistently, Santos et al. demonstrated that patients with high Gleason score tumors had elevated circulating levels of miR-221 and miR-7, and this was associated with early development of CRPC (10 vs. 46 months) [39]. Additionally, high miR-7 was predictive of lower OS.

Other Circulating PrCa Prognostic MiRNA Biomarkers

Several other miRNAs of prognostic relevance are uncovered in circulation of PrCa patients. Selth et al. demonstrated that elevated levels of serum miR-141, miR-146b-3p, and miR-194 were associated with biochemical recurrence post-

prostatectomy [40]. In a validation cohort, miR-146b-3p and miR-194 were predictive of disease progression. Serum miR-21 is elevated in CRPC compared to BPH patients, and the levels are associated with resistance to docetaxel therapy [41]. Levels of miR-375, miR-1246, and miR-1290 have also been associated with CRPC prognosis in a Cox regression analysis [42]. Higher circulating levels of miR-375 and miR-1290 were significantly associated with OS ($p < 0.004$). Predictive performance was significantly improved when the two miRNAs were included in a clinical prognostic factor-based model of CRPC (AUROCC ranged from 0.66 to 0.73). DNA hypermethylation of tumor suppressor miR-205 is significantly predictive of biochemical recurrence [43]. SNPs in miRNA (rs2043556 in miR-605) and in miRNA target genes (rs3737336 in *CDON*) have been related to biochemical recurrence after prostatectomy ($p < 0.05$). In silico work indicates that SNP rs3737336 interferes with targeting of a number of miRNAs (miR-181a, miR-181b, miR-181c, miR-181d, miR-4262, miR-5007 [44]).

Circulating PrCa Predictive MiRNA Biomarkers

First-line chemotherapy for CRPC is docetaxel that has a response rate of ~50 %. Because half of all such patients do not respond to this therapy, there is a need to identify biomarkers that will enable stratification of patients who will have optimum treatment outcomes while avoiding unnecessary toxicities in nonresponders. Some miRNAs have demonstrated potential for use in predicting therapy response according to biochemical and other clinical parameters. Serum miR-21 is a possible predictor of docetaxel response. MiR-21 is especially elevated in patients with CRPC who are unresponsive to docetaxel, in comparison with patients with androgen-dependent PrCa, localized disease, and BPH [41]. Serum miR-141 was studied in reference to its predictive ability in patients receiving chemotherapy, hormonal therapy, and biotherapy (kinase inhibitors or vaccines). Plasma miR-141 was able to predict clinical disease progression (OR 8.3) under these therapeutic regimens with a sensitivity of 78.9 % and specificity of 68.8 %. Additionally, the elevated levels correlated with CTCs ($p < 0.001$) and PSA levels ($p < 0.001$) [37]. Of 365 miRNAs evaluated, miR-141, miR-200a, miR-200c, miR-210, and miR-375 were elevated in sera from patients with mCRPC compared to age-matched controls [45]. MiR-210, a transcriptional target of hypoxia signaling, was induced and released by activation of HIFα signaling and serum levels correlated with treatment response as evidenced by PSA dynamics. Lin et al. identified 46-candidate miRNA using global profiling of docetaxel-resistant and sensitive cell lines. Circulating levels of 14 of these miRNAs were associated with PSA response and overall survival [46]. High pretreatment miR-200 family and low or normal posttreatment miR-17 family members were associated with non-response (indicated by PSA levels) and poor survival. In multivariate analysis, pretreatment miR-200b and posttreatment miR-20a levels (as well as pretreatment hemoglobin and visceral metastasis) were independent predictors of overall survival of patients on docetaxel chemotherapy.

12.4.5.2 Circulating PrCa LncRNA Biomarkers

A number of lncRNAs show deregulated expression in primary PrCa tissue samples, however, their role in circulation remains to be fully explored. These include PCA3, MALAT1, prostate cancer gene expression marker 1 (PCGEM1), prostate cancer noncoding RNA 1 (PRNCR1), second chromosome locus associated with prostate 1 (SCHLAP1), prostate cancer-associated ncRNA transcripts 1, 6, 7, and 18 (PRCAT1, 6, 7, and 18), PTENP1, GAS5, and ANRIL, among several others. The most extensively studied lncRNA for PrCa detection is PCA3, which is overexpressed over 60-fold in ~95 % of PrCas. As a circulating diagnostic biomarker, MALAT1, which is overexpressed in PrCa tissues, achieved a dismal sensitivity comparable to PSA of 58.6 % (AUROCC of 0.767) [47]. However, MALAT1 overexpression is associated with poor prognostic variables such as high TNM stage, high Gleason scores, and PSA levels (>20 ng/ml). Elevated tissue levels of MALAT1 were associated with CRPC, increased tumor growth, invasion, and metastasis. Additionally, PRCAT1, 6, and 7 may be associated with PrCa prognosis. PRCAT1, which controls cell proliferation, is overexpressed in high Gleason grade (≥7) and mPrCa, and PRCAT6 and 7 that control cell growth, demonstrate increased expression with progressive disease stage from primary to metastatic PrCa. Also, SCHLAP1, which is overexpressed in PrCa, is associated with aggressive disease, biochemical recurrence, and clinical PrCa progression.

LncRNAs also appear to relate to androgen receptor status. For example, PCGEM1 and PRNCR1 are overexpressed in PrCa tissues and appear to regulate alternative splicing of AR as well as AR-mediated gene expression [48]. AR signaling also appears to induce PRCAT18 expression [49], and CBR3 antisense RNA1 (CBR3-AS1) may also alter the activity of AR [50].

12.4.6 Circulating PrCa Protein Biomarkers

Serum protein biomarkers are explored to improve the screening for PrCa. While several are uncovered, promising leads include autoantibodies and panels of kallikreins. Adding to this are novel proteomic approaches that are used to mine the PrCa serum proteome/peptidome.

12.4.6.1 Circulating Autoantibodies as PrCa Biomarkers

Alpha-methylacyl-CoA racemase (AMACR) converts C27-bile acyl-CoAs and pristanoyl-CoA between their R- and S-stereoisomers. The S-stereoisomer is required for degradation of these molecules by beta-oxidation. AMACR, which localizes to mitochondria and peroxisomes, is overexpressed in PrCa. AMACR autoantibodies in serum could differentiate men with PrCa from healthy controls at

a better performance than PSA (AUROCC of 0.789 vs. 0.492 for PSA) [51]. This was suggested to complement PSA in the detection of clinically significant PrCa, especially those in the intermediate PSA zone. Early stage PrCa detection using autoantibodies has shown potential in some other proof of principle studies. In a diagnostic case–control study, serum samples from 119 patients with PrCa and 138 controls were divided into training and validation sets and subjected to phage microarray analysis. A 22-phage peptide detector achieved a sensitivity of 81.6 % and specificity of 88.2 % in PrCa detection in the validation cohort. This panel of autoantibodies performed better (AUROCC of 0.93) than serum PSA (0.80) in this cohort [52]. In another study, sera from an initial 20 PrCa patients and 20 healthy controls were incubated on expression clone array containing >37,000 recombinant proteins. This enabled identification of 174 PrCa-specific autoantibodies enriched for nuclear, RNA-associated, and cytoskeletal proteins. These biomarkers were then validated on microarrays using independent sera from 40 PrCa patients and 40 control men with elevated PSA but negative for PrCa and 40 healthy controls. The discriminating power of cancer vs. controls achieved an AUROCC of 0.71, with TTLL12 being the highest ranked discriminating antigen [53].

12.4.6.2 Circulating Kallikreins as PrCa Biomarkers

Two European studies have explored the use of kallikreins (KLKs) in panels in an attempt to improve the accuracy of the PSA test. Four KLK biomarkers (total, free, and intact PSA (KLK3) and KLK2) were assayed in sera from 740 men undergoing biopsy in the first round of the European Randomized Study of Screening for PrCa. Two diagnostic models, age and total PSA (lab model) and age, total PSA, and DRE (clinical model), were enhanced by the addition of the other KLKs. Thus, the addition of free PSA, intact PSA, and KLK2 increased the diagnostic accuracy from AUROCC of 0.68 to 0.83 for the lab model and from 0.72 to 0.84 for the clinical model [54]. An independent replication of the previous study was conducted on a large representative population-based cohort including 2914 previously unscreened men scheduled for biopsy consequence to elevated PSA. Prostate cancer was detected in 28 % (807) of these men. The cohort was randomly divided into a training and validation sets, and the levels of biomarkers were compared with biopsy outcomes. Consistent with their previous findings, the 4-biomarker panel improved PrCa detection in both models with AUROCC change from 0.64 to 0.76 for the lab model and 0.70 to 0.78 for the clinical model ($p < 0.001$ for both models). Application of this 4-kallikren biomarker panel will reduce the majority of unnecessary biopsies [55].

12.4.6.3 Circulating IL-6 and TGFβ1 as PrCa Biomarkers

A number of other emerging serum markers including IL-6 and TGFβ1 have proven clinically useful for PrCa management. IL-6 and its soluble receptor (sIL-6R) are

elevated in PrCa cell lines and tissues. Elevated serum levels have shown prognostic relevance in a number of validated studies. They have demonstrated significant association with metastasis and hormone-refractory disease [56]. Drachenberg et al. demonstrated significantly elevated levels of serum IL-6 in patients with hormone-refractory PrCa (5.7 $\pm$ 1.9 pg/ml) in comparison with patients with BPH, chronic prostatitis, lPrCa, and normal controls ($p < 0.01$) [57]. Serum IL-6 was compared with PSA, free PSA, and free/total PSA in men suspected of having PrCa prior to biopsy [58]. While IL-6 levels were not statistically different among men with PrCa, BPH, HGPIN, and chronic prostatitis, serum IL-6 levels however significantly differentiated patients with poorly differentiated disease as per Gleason score ($4 + 3 = 7$ and >7) from those with moderately differentiated PrCa ($3 + 4 = 7$ and <7; $p = 0.007$).

Apart from IL-6, another serum biomarker associated with advanced stage disease is TGFβ1. Immunohistochemistry study implicated TGFβ1 overexpression with higher grade, local invasion, distant metastasis, and biochemical recurrence. An immunoassay detection of plasma TGFβ1 associated high levels with invasive PrCa [59]. Other investigators have confirmed these studies, demonstrating that elevated serum TGFβ1 levels are significantly associated with extracorporeal spread, seminal vesicle invasion, and biochemical recurrence, consistent with the IHC evidence [60].

In view of the validated impressive performances of serum IL-6 and TGF-β1 in identifying patients with aggressive PrCa, Kattan et al. performed a validation study using preoperative plasma from patients [61]. They concluded: *...that pretreatment plasma levels of IL6SR and TGF-beta1 improved the ability to predict biochemical progression by a prognostically substantial margin. A nomogram including the pretreatment levels of these molecular markers, along with standard clinical markers, has been developed and internally validated*. Validation of these biomarkers has also been demonstrated by multi-institutional studies [62].

12.4.6.4 Circulating PrCa Proteomic Biomarkers

The search has been on for the ideal biomarkers for PrCa management. Optimal screening biomarkers, and importantly predictive biomarkers of treatment and tumor behavior (indolent vs. aggressive), are all still needed. Hence efforts at uncovering these in the secretome or degradome using proteomic approaches are ongoing.

Circulating PrCa Diagnostic Proteomic Biomarkers

Serum from 154 men with PSA between 2.5 and 15 ng/ml were subjected to weak cation exchange protein chip (WCX2) analysis. A discriminatory model was developed and tested on 91 independent samples achieving a sensitivity of 100 % at 67 % specificity [63]. Using a lectin immunoabsorbant assay, glycosylated PSA was measured in sera from men with PrCa and controls. This assay with AUROCC of 0.71 was more superior to serum percent-free PSA (AUROCC 0.54) in PrCa

detection [64]. Using 2D DIGE analyses of serum samples from PrCa patients enabled identification of protein spots that could differentiate patients with Gleason score 5 from those with score 7 tumors. Two characterized proteins, pigment epithelial-derived factor (PEDF) and zinc-α2-glycoprotein, were validated in a large cohort of independent samples, and the findings suggest PEDF has potential for early PrCa detection [65]. Another proteomic approach, gel- and lectin-based proteomics, was used to profile sera from PrCa and BPH patients. Differentially expressed proteins between the two groups were apolipoprotein AII, complement C3β chain fragment, inter-α-trypsin inhibitor heavy chain 4 fragments, transthyretin, α-1 antitrypsin, and high molecular weight kininogen light chain.

Circulating PrCa Recurrence Prediction Proteomic Biomarkers
The ability to stratify men diagnosed with PrCa into indolent and aggressive categories will certainly save procedural and psychological harm as well as escalating healthcare costs. Proteomic approaches to identify such markers are also been pursued. Serum samples from men with indolent and aggressive PrCa according to Gleason scores were analyzed by SELDI-TOF MS. Twenty-six protein peaks could separate the indolent from the aggressive group at a sensitivity of 73.3 % and specificity of 60 % ($p < 0.05$). Additionally, 18 protein peaks could differentiate PrCa patients with recurrences from those with nonrecurrent disease at a sensitive and specificity of 70 % and 62.5 %, respectively [66]. Rehman et al. analyzed pooled samples from four categories of men with prostatic diseases that were followed for 5 years [67]. The groups included men diagnosed with only BPH, lPrCa with no evidence of progression (nonprogressing), lPrCa with biochemical evidence of progression (progressing), and those with bone metastasis (metastatic). Twenty-five proteins could significantly separate the progressing from nonprogressing group, while 23 proteins could significantly differentiate between lPrCa-progressing from the metastatic group. These protein panels included eEF1A1, which was more highly expressed by osteoclasts in the vicinity of metastatic cancer cells compared to controlled osteoclasts ($p = 0.0353$, Mann Whitney U). Chip-based affinity proteomics also enabled identification of protein signatures that could accurately classify biochemically defined risk groups of men who underwent PSA testing [68].

12.4.7 Circulating PrCa Metabolomic Biomarkers

One-carbon metabolism is implicated in carcinogenesis because of its involvement in nucleotide biosynthesis, DNA methylation, and repair processes. A number of the components including cofactors of these pathways have been studied in blood samples from PrCa patients as diagnostic and prognostic biomarkers. These molecules include folate, B vitamins, choline, methionine, betaine, cysteine, serine, glycine, and sarcosine, among others.

N-methylglycine or sarcosine is a natural amino acid generated from glycine by glycine *N*-methyltransferase. Sarcosine was identified by metabolomic studies of PrCa tissue samples and implicated in the pathogenesis of this disease by its ability to induce invasive phenotypes in benign prostate epithelial cells and possibly conferring invasive properties on PrCa cells. Consistent with these findings, circulating sarcosine is also emerging as a potential risk factor biomarker of PrCa. Sarcosine levels in blood and urine could accurately stratify men with benign prostates, those with lPrCa and mPrCa [69]. Jentzmik et al. analyzed sarcosine levels in urine and failed to detect any differences in the levels between men with PrCa and benign disease or those with aggressive disease as suggested by other studies [70]. Lucarelli et al., however, reported the enhanced detection of PrCa in men with PSA of <4 ng/ml using serum sarcosine levels [71]. In a follow-up study, they further demonstrated that serum sarcosine levels were significantly more elevated in men with mCRPC than those with non-mCRPC (0.81 vs. 0.52 nmol/ul, $p < 0.0001$). In a multivariate analysis, this biomarker was an independent predictor of PFS and OS [72]. Buttressing these findings, a prospective study of serum sarcosine content as PrCa biomarker in the PLCO trial identified a significant association between increased serum sarcosine and risk of PrCa [73]. This large-scale study included 1122 cases (813 with non-aggressive PrCa and 309 with aggressive disease) and 1112 controls. Sarcosine was quantified using high-throughput liquid chromatography–MS. An increasing serum sarcosine level was associated with significantly elevated risk of developing PrCa, and this risk was stronger in men with diabetes, and those with non-aggressive PrCa. A Norwegian nested case–control study of 3000 cases and 3000 controls from the JANUS cohort indicated high sarcosine and glycine concentrations were rather associated with moderately reduced risk of PrCa and so was the ratio of glycine/serine (OR 0.74, 95 % CI 0.69–0.85, $p < 0.001$) [74]. These inconsistent results likely reflect technical issues with quantifying sarcosine levels in blood and/or differences in study populations making each finding not generalizable.

Other one-carbon metabolites have produced mixed results. Johansson et al. analyzed a panel of seven B vitamins and their metabolites in relation to their role in PrCa risk [75]. A large-scale nested study of the Northern Sweden Health and Disease Cohort prospective samples from 561 cases and 1034 controls was included in the study. Of seven biomarkers including betaine, cysteine, methionine, vitamin B2, B6, choline, and MMA, only choline, vitamin B2, and MMA were associated with PrCa risk. Elevated choline and vitamin B2 and lower levels of MMA were risk factors for PrCa. There is certainly the need for standardized protocol development for measuring these metabolites, especially sarcosine.

12.4.8 Circulating PrCa Cells

Circulating PrCa cells (CPrCaCs) are useful for prognostic determination of disease progression as well as predictive use in treatment selection and other clinical

decision-making in men with metastatic CRPC. Circulating PrCa cells, defined by the CELLSEARCH® criteria, were assayed in men with mPrCa. The number of CPrCaCs correlated with disease progression, being virtually absent in controls (mean number 0.8/7.5 ml of blood), compared to patients with lPrCa (5.9/7.5) and mPrCa (46.6/7.5). High CPrCaC counts (68.5/7.5 ml of blood) were associated with disease progression [76]. This group subsequently established antisense RNA libraries from immunomagnetically enriched CPrCaCs from patients with HRPC [77]. These libraries are useful for multigene characterization of CPrCaCs in HRPC patients. PrCa-specific genes including *PSA, PSMA, AR, KLK2, EGFR*, and prostate-specific gene with homology to a protein receptor were abundantly expressed in these libraries. In another study by this group, a substantial number of dead CPrCaCs were observed in treated PrCa patients, and similar events were demonstrated in paclitaxel treated LNCaP cells spiked into normal blood [78]. This finding suggests the evaluation of CPrCaCs may be useful for treatment response monitoring.

It has been demonstrated that high baseline CPrCaCs are associated with aggressive tumors, and this event correlates with increased LDH and PSA levels, low albumin and hemoglobin levels, as well as PSA doubling time <3 months. CPrCaCs ≥ 5 predicted shorter median OS, and importantly CPrCaCs >50 have significantly been associated with decreased median OS, and more so than CPrCaCs between 5 and 50 (6.3 vs. 21.1 months). Multivariate analysis found CPrCaCs ≥ 5 to be an independent prognostic factor. While so, it was also uncovered that a drop of CPrCaCs ≥ 30 % was associated with improved OS [79]. High CPrCaC counts were more often associated with patients who had bone marrow involvement compared to patients with soft tissue metastasis. Also CPrCaCs were high in patients on chemotherapy compared to those without. Baseline CPrCaCs strongly correlated with survival, and this prediction was improved when used in combination with PSA and albumin levels [80]. In this study, CPrCaC counts were dichotomized at cutoff levels of five CPrCaCs /7.5 ml of blood. High pretreatment and posttreatment CPrCaCs (above cutoff) were associated significantly with shorter median OS than low CPrCaCs. Posttreatment CPrCaCs predicted OS much better than PSA levels and also much sooner than imaging or PSA decreases. Moreover, patients with high baseline CPrCaC count and who later converted to low counts (<5/7.5 ml of blood) and those that converted from low to high counts had better and worse OS, respectively. CPrCaCs demonstrated superior accuracy as independent predictor of survival in men with CRPC. This compelling data led to the FDA approval of the CELLSEARCH® system for management of CRPC [81]. Because of the fact that dichotomous CPrCaC numbers yielded misleading information needed for patient management, especially with regard to CPrCaC conversions between baseline and posttreatment CPrCaCs, the de Bono et al. data was reanalyzed with CPrCaCs viewed as continuous variables. This reanalysis revealed that high CPrCaCs (and other biochemical variables) were predictive of death form PrCa. Importantly, changes in CPrCaC counts were strongly associated with risk of death. PSA levels were not that informative of risk of death in this series [82].

12.4.8.1 CPrCaCs as Monitoring Biomarkers

Eschwege et al. used PCR targeting *PSA* and *PSMA* mRNAs to evaluate the prognostic relevance of CPrCaCs in men with organ-confined PrCa [83]. Five-year follow-up revealed that preoperative CPrCaCs were independent predictors of tumor recurrence, and also that intraoperative tumor cell release had no effect on disease progression. In a pilot phase II trial of men with CRPC who received abiraterone acetate following docetaxel treatment, CPrCaC counts decreased following therapy. While this study was not well powered, the findings triggered a phase III trial involving 1195 men with CRPC. The observations from this study were consistent with the phase II trial in that CPrCaC counts significantly correlated with survival outcomes. Thus, baseline CPrCaCs and CPrCaC conversions of these men on abiraterone and low-dose prednisone after docetaxel administration predicted OS [84]. Consistent with bone being the preferred "soil" for PrCa cells, CPrCaCs are much higher in patients with bone metastasis compared to those with visceral organ involvement [79, 80, 82]. These findings indicate the potential of using CPrCaC enumeration in monitoring for early detection of patients likely to have bone involvement.

12.4.8.2 Other CPrCaC Biology

Medeiros et al. found association of endothelial nitric oxide synthase polymorphism ecNOS4ab (genotype *ab*/*aa*) to be associated with CPrCaC presence in blood of PrCa patients, especially patients under age 67 ($p = 0.003$), and lPrCa patients ($p = 0.012$). Putative explanation for this observation is that the allele promotes CPrCaC survival in blood [85]. After immunomagnetic enrichment, *HER2*/*neu* expressing CPrCaCs were detected in 54 % of mPrCa compared with 9.6 % in lPrCa patients, suggesting the usefulness in targeting *HER2*/*neu* expressing mPrCa cells in these patients [86].

12.5 PrCa Extracellular Vesicles

In PrCa, the terms exosomes and prostasomes are sometimes used interchangeably, although these two vesicles have structural and size differences that also overlap (maybe because both are secreted via MVBs). The size range of prostasomes (40–500 nm) overlaps with exosomes (40–100 nm), and both contain CD9 and CD63. But prostasomes have unique membrane composition enriched for cholesterol (cholesterol/phospholipid ratio is 2:1) and also possess specific markers such as CD39, CD46, CD55, CD59, Anx1, and PSA. Prostasomes are more enriched in seminal fluids and are assigned physiologic reproductive functions. PrCa-derived microvesicles are enriched with PrCa-associated proteins such as PSA, FASN,

PTEN, PSMA, and MHC, as well as RNAs including PCA3, TMPRSS2-ERG, and several miRNAs in addition to their unique lipids (glycosphingolipids, sphingomyelin, phosphatidylserine, and cholesterol). The clinical utility of circulating PrCa-derived microvesicles has been addressed in a few studies primarily focused on their amount and cargo. The number of circulating exosomes has shown potential as PrCa biomarkers. Tavoosidana et al. used a modified proximal ligation assay, whereby antibodies are first used to immobilize the microvesicular targets followed by four additional targeted antibodies with DNA strands [87]. Using the term prostasomes, these microvesicles were increased in serum from PrCa patients before surgery compared to controls and may have prognostic association as well. Two independent analyses indicated that plasma microvesicular content was 2.5–7-fold higher in PrCa patients than controls. Tumor aggressiveness was also predictable using plasma microvesicular levels, as patients with Gleason scores 7 and higher could be separated from those with lower scores (≤ 6). Exosomal survivin has also been assayed in plasma and sera from men with PrCa, PrCa relapse, BPH, and healthy controls. Exosomal survivin was significantly higher in plasma from cases than controls and was independent of tumor Gleason score (low or high) suggestive of its early detection capability [88].

The role of PrCa-derived circulating exosomal miRNA has been addressed by other studies as well. The work of Bryant and colleagues identified a number of miRNA in PrCa microvesicles [30]. Consistent with other findings, exosomal levels were significantly higher in cancer patients than controls. A set of 12 miRNAs (miR-107, miR-130b, miR-141, miR-181a-2, miR-2110, miR-301a, miR-326, miR-331-3p, miR-432, miR-484, miR-574-3p, and miR-625) could differentiate men with PrCa from controls. MiR-141 and miR-375 were associated with mPrCa as validated in serum exosomes from an independent cohort of patients with recurrent or nonrecurrent disease. Additionally, lPrCa could be distinguished from normal controls by miR-107, miR-141, miR-181a-2, miR-2110, miR-301a, miR-326, miR-432, miR-484, miR-574-3p, and miR-625, while mPrCa was separable from lPrCa by miR-582-3p, miR-20a, miR-375, miR-200b, miR-379, miR-572, miR-513a-5p, miR-577, miR-23a, miR-1236, miR-609, miR-17, miR-619, miR-624, miR-198, and miR-130. Huang et al. examined plasma exosomal miRNA as prognostic biomarkers in men with CRPC [42]. An initial cohort of CRPC patients was analyzed to identify candidate miRNAs, which were then validated in independent samples from men with CRPC. Elevated miR-1290 and miR-375 had significant association with poor OS ($p < 0.004$).

12.6 Summary

- PrCa is the most commonly diagnosed male cancer.
- The primary risk factor for PrCa is age, such that the risk increases after age 50. There are multiple other risk factors such as lifestyle and genetic composition (some SNPs confer elevated risk).

- Screening for PrCa therefore begins at age 50 with PSA testing and digital rectal examination.
- Because of the poor performance of the PSA test, issues with overdiagnosis and overtreatment have been major concerns in PrCa screening.
- Although many cases of PrCa are indolent, some become refractory to androgen deprivation therapy, leading to the development of CRPC, which can be lethal.
- Noninvasive accurate, safe, and effective biomarkers of PrCa are being sought after.
- The molecular pathology of PrCa is well charted.
- The known molecular pathologic changes, as well as novel biomarkers, are targeted in circulation.
- Circulating epigenetic (e.g., *GSTP1*), genetic (e.g., MSA), mRNA (e.g., *hTERT*), and noncoding RNA alterations are common biomarkers of PrCa.
- The altered metabolic signatures of PrCa are demonstrable in circulation.
- Circulating PrCa cells are of prognostic, predictive, and therapy monitoring potential.

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Chapter 13
Ovarian Cancer Biomarkers in Circulation

Key Topics

- Molecular pathology of ovarian cancer (OvCa).
- Circulating OvCa biomarkers.
- Circulating OvCa miRNA biomarkers.
- Circulating OvCa protein biomarkers.
- Circulating OvCa cells.

Key Points

- OvCa is mostly a disease of postmenopausal women, but as many as 30 % of cases occur in women under the age of 50. The incidence is on the rise; however, tumors detected early have good prognosis. Noninvasive biomarkers for early detection of OvCa in perimenopausal and postmenopausal women should improve the outlook for OvCa.
- Serum CA125 remains a useful biomarker of OvCa. Its utility in multivariate index assays increases the accuracy of OvCa risk prediction.
- Novel circulating OvCa biomarkers, including miRNAs, proteomic discoveries, as well as circulating OvCa cells add to the clinical armamentarium of OvCa biomarkers.

13.1 Introduction

Globally, over 239,000 women were estimated to be diagnosed with OvCa in 2012, while in the same year as many as 140,000 women died from the disease. This mortality rate is on the rise, because in 2010, 160,000 women died from OvCa,

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which was up from 113,000 in 1990. OvCa is the most lethal gynecologic cancer, and globally is the 7th cause of cancer-related deaths. In the US alone, the 2016 estimated cases and deaths are 22,280 and 14,240 respectively. There are geographic variations in the incident rates, with rates being higher in the more developed than the less developed parts of the world. For example, the rate of 11.7/100,000 women in the UK contrasts sharply with that of 4.1/100,000 for other parts of the world.

The 5-year survival rate for women diagnosed with OvCa has increased over the past decades due to improved gynecologic surgical techniques, coupled with combination therapies, but not due to early detection. While it is established that early detection of organ-confined tumors (stage I) has the best prognosis, OvCas are rarely detected early. Indeed, only ~15 % are localized cancers, with the vast majority having some regional spread (18 %) or worse still distant metastasis (~60 %). The survival rate can be as high as 92 % for organ-confined early-stage disease. This survival rate drops significantly to under 30 %, when cancers spread to the peritoneum or distant organs. The need for early detection biomarkers is thus obvious. Because there are no recommended screening procedures, coupled with the need for early and appropriate treatment, the "risk malignancy index" (RMI) was established in the early 90s. This risk index includes ultrasound detection of pelvic masses, elevated serum CA125, and menopausal status. While RMI has a good sensitivity (71 %–88 %) and a high specificity (74 %–97 %), only women considered to be above average risk receive such periodic screening, leaving many, especially premenopausal women without any screening, though OvCa occurs in ~30% of this population. Indeed, while commonly diagnosed in women above the age of 55, the incidence rises from about age 35–39.

CA125 is the most commonly used noninvasive screening biomarker. Overall, CA125 is elevated in about 80% of women with OvCa. This rate is ~60% in stage I and up to 90% in advanced stage III/IV diseases. Thus, its use in early detection has shortcomings. However, because it has established utility in monitoring chemotherapy response, the FDA has approved this biomarker for such intended use. However, the CA125 assay has limitations. Endometriosis, menstruation, and benign uterine lesions in premenopausal women can elevate circulating levels of CA125. There is still therefore a need for better noninvasive biomarkers with increased accuracy and companion diagnostic ability for OvCa.

13.2 Screening Recommendations for OvCa

There are currently no screening programs or recommendations for OvCa detection in the at-risk population. In women with established family history of the disease, periodic CA125 measurements coupled with transvaginal ultrasound are recommended. Current utility and benefits of CA125 biomarker in OvCa include:

- Monitoring for disease recurrence after treatment. Elevated levels indicate recurrence in up to 70 % of cases.

- Monitoring for effectiveness of chemotherapy. In 90 % of cases, persistent elevated levels are indicative of disease presence. Response should be associated with a decline.
- Used to assess disease burden with over 90 % accuracy.

The NACB also recommends CA125 measurements for:

- Differential diagnosis of pelvic masses.
- Early detection of cancer in combination with transvaginal ultrasound in people with familial predisposition.
- Detection of recurrence.
- Monitoring therapy response.
- Prognosis.

Other preventive measures of OvCa are the provision of prophylactic surgery to *BRCA1* and *BRAC2* carriers and kindred of Lynch syndrome who are at high risk for the disease. While the vast majority (~75 %) of OvCas are diagnosed in postmenopausal women, as many as 30 % of tumors are detected in people under the age of 55. It would therefore be helpful to develop simple cost-effective tests that can be used to screen all women after the age of 40.

13.3 Molecular Pathology of OvCa

13.3.1 Origins of OvCa

OvCas are from diverse cellular origins; however, the most common ovarian tumors are of epithelial origin (EOCs). These tumors are classified histologically based on the WHO/FIGO recommendations into the following different types: serous, mucinous, endometrioid, clear cell, and transitional types. The majority of EOC are of serous histology. The different types harbor shared and unique molecular changes, and also have different clinical behaviors. Epithelial OvCas are also heterogeneous in regard to their origins, which is reminiscent of the anatomic relationship and location of the ovaries. The putative precursor lesions of surface EOCs are:

- In situ lesions of the surface epithelium or surface epithelial inclusion glands.
- Benign epithelial tumors.
- Endometriosis.

However, the most common subtype of OvCa, high-grade serous carcinoma (HGSC), is mostly of extra-ovarian source. Various molecular genetic data suggest the majority (~60 %) of HGSCs develop from intraepithelial lesions in the fallopian tube, and involve the ovaries secondarily. Additionally, some HGSCs may arise from cortical inclusion cysts. It is thus becoming evident that the majority of OvCas originate from outside the ovaries.

13.3.2 Dualistic Molecular Subtypes of OvCa

Irrespective of their origins, from actionable clinical perspective, a dualistic model recognizes low-grade (type I) and high-grade (type II) EOCs. These two have different histologic grades and types of molecular events (Fig. 13.1).

- Type I are mostly low-grade serous EOCs. They are indolent tumors that present early (at stage I). They develop in a stepwise predictive fashion from atypical proliferative or borderline epithelial tumors. They also include mucinous, endometrioid, clear cell, and malignant Brenner tumors. Molecularly, these tumors are more genetically stable and rarely harbor *TP53* mutations. They mostly activate the MAPK, PI3K, and WNT/β-catenin signaling pathways due to mutations in *BRAF, KRAS*, and *ERBB2* (serous); *KRAS* (mucinous); *PTEN* and *CTNNB1* (endometrioid); and *PIK3CA* (clear cell).
- Type II EOCs are high-grade serous carcinomas. These are aggressive tumors that present at advanced stage. They are commonly associated with chromosomal aberrations and *TP53* mutations. Additionally, *BRCA* mutations are associated with the development of these fallopian tube-derived HGSCs. Other aggressive OvCas are malignant mixed mesodermal tumors (carcinosarcomas) and undifferentiated carcinomas.

This histologic delineation is important for OvCa biomarker exploration, disease prevention, screening, and treatment approaches.

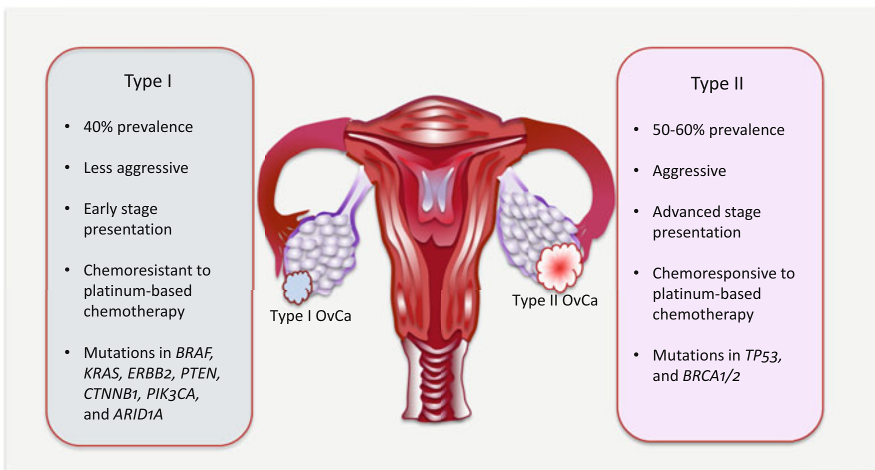

Fig. 13.1 Features of the two major subtypes of OvCa

13.4 Circulating OvCa Biomarkers

Notwithstanding its limitations, CA125 remains a useful noninvasive biomarker of OvCa. To improve upon OvCa management, efforts at uncovering novel biomarkers including ccfDNA levels, as well as genetic and epigenetic alterations in ctDNA are sought after. Additionally, alterations in the levels of miRNA and novel proteins are pursued. An emerging class of prognostic biomarkers is circulating OvCa cells.

13.4.1 Circulating Cell-Free Nucleic Acid Content as OvCa Biomarkers

The promising role of ccfDNA in the diagnosis, treatment prediction, and prognosis of women with OvCa has been demonstrated by a number of studies. In a proof of principle study, female nude mice were injected intraperitoneally with OvCa cell line, HeyA8, and it was demonstrated that ccfDNA levels increased with increasing tumor burden, and also modulated with chemotherapy. Thus, tumor ccfDNA was elevated at 63 % above baseline in 24 h following docetaxel therapy, and then declined to below 20 % of baseline in 72 h, with further decline to below 83 % of baseline in 10 days after therapy [1]. In a follow-up study, ccfDNA was quantified in women with OvCa and healthy controls targeting *GAPDH, ACTB*, *and HBB* [2]. In this cohort of women with advanced stage OvCa (stage III/IV), ccfDNA was significantly higher than in healthy control women at all three genomic loci examined. Zachariah et al. also demonstrated the potential diagnostic utility of ccfDNA in women with OvCa [3]. They revealed that ccfDNA from both nuclear and mitochondrial genomes were significantly increased in cancer patients compared to both healthy controls and women with benign ovarian diseases. Additionally, there were significant differences in the levels of mtDNA between women with OvCa and those with endometriosis. CcfDNA has roles in predicting outcomes in OvCa women on therapy. Wimberger et al. revealed that residual tumor load in OvCa patients on platinum-based chemotherapy contributes to increases in ccfDNA [4]. There was also a significant association between residual tumor volume (>1 cm) after surgery and the levels of serum DNA. The increases in ccfDNA as well as the presence of DTCs were significantly associated with increased relapse and poor overall survival. In multi-resistant OvCa women on bevacizumab-targeted therapy, ccfDNA significantly correlated with PFS ($p = 0.0004$) and OS (0.005) in both univariate and multivariate analyses. High ccfDNA was a positive predictor of poor prognosis [5]. Circulating cfDNA, p53 antibodies, and *KRAS* mutations could predict the outcomes in women with different types of OvCa. Circulating antibodies against p53 and ccfDNA were frequently associated with women who had HGSCs, and this predicted worse overall survival. Similarly, circulating *KRAS* mutations were common in women with mucinous OvCa and was an indicator of worse OS [6].

13.4.2 Circulating OvCa Epigenetic Biomarkers

13.4.2.1 Circulating OvCa Diagnostic Epigenetic Biomarkers

Methylated genes are explored as circulating diagnostic biomarkers of OvCa. Ibanez et al. screened ovarian tumors and matched preoperative sera/plasma and peritoneal fluids for *RASSF1A* and *BRAC1* methylation status [7]. Hypermethylation of one or both genes was demonstrated in 68 % of tumors. They then expanded coverage by including the following genes: *APC, CDKN2A, DAPK,* and *CDKN2A/ARF,* which enabled the remaining tumors to be identified. Identical methylation pattern was present in 82 % of serum/plasma samples (including 76.5 % of stage I disease samples). Peritoneal fluid was positive in 93.3 % of the samples, including samples from patients with tumors that were scored conventionally as atypia or negative by cytology. Microarray-based screening of 58 genes, and using the most differentially methylated genes in naïve Bayesian analysis enabled 10 genes to be identified for further study. The sensitivity and specificity in tissue samples were 69 % and 70 %, respectively. A 5-gene panel analyzed in plasma samples achieved a sensitivity of 85 % and specificity of 61 %. This assay demonstrates diagnostic potential [8]. In a follow-up study, Levenson's group expanded their proof-of-principle study in an attempt to differentiate women with healthy ovaries from those with OvCa and benign ovarian diseases, and to possibly differentiate benign ovarian diseases from cancer using methylation of a number of gene sets in ccfDNA. Methylation of *RASSF1A, CALCA*, and *EP300* differentiated cancer from healthy controls at a sensitivity of 90% and specificity of 86.7 %. Similarly, methylation of *BRAC1, CALCA,* and *CDKN1C* differentiated women with benign ovarian disease from healthy controls at a sensitivity of 90 % and specificity of 76.7 %. Finally, *RASSF1A* and *PGR-PROX* methylation in ccfDNA differentiated cancer from benign ovarian disease [9]. Bondurant et al. used a sensitive assay that enabled detection of 1 methylated among 100,000 unmethylated alleles [10]. Using this assay, *RASSF1A* was methylated in 51 % of invasive serous OvCa samples, and was concordant in all available matched preoperative serum samples. Serial serum sampling enabled therapy response monitoring that was detectable as fluctuations in *RASSF1A* methylation in some patients, reflective of disease status.

13.4.2.2 Circulating OvCa Prognostic Epigenetic Biomarkers

In addition to tumor grade and histologic cell type, the cytologic examination of peritoneal fluid is part of the staging strategies of OvCa. Women with positive peritoneal fluid cytology have poor prognosis regardless of FIGO stage. The inherent issues with cytology call for better prognostic biomarkers or complementary assays to cytology. As a proof-of-principle study to identify sensitive prognostic methylation biomarkers, peritoneal fluids were collected at surgery from women

with OvCa, who also received platinum-based chemotherapy after surgery. Fifteen genes were selected for study. Women with fewer methylated genes had poor or shorter overall survival evidenced by univariate and multivariate analysis, independent of age, FIGO stage, or grade. Thus, methylation in genes from this series may confer sensitivity to platinum-based chemotherapy [11].

13.4.3 Circulating OvCa Genetic Biomarkers

Studies of genetic changes in circulation of OvCa patients have mainly been proof of concept discovery studies. Serum and peritoneal fluid DNA samples from OvCa patients were analyzed for known tissue-specific genetic changes. Six polymorphic markers on four chromosomes were assessed for LOH detection, which were positive in 85 % of serum and 63 % of peritoneal fluid samples. Half of the LOH observed in tissue samples were specifically detected in sera [12]. Zhang et al. questioned the relevance of LOH on chromosome 3p14 in serum DNA samples from patients with OvCa. Four polymorphic markers (D3S1029, D3S1228, D3S1300, D3S1481) were employed, and the detection frequency of at least one alteration was 78 %. Indeed, 45 % of serum samples had more than a single MSA. LOH was associated with advanced stage disease [13]. LOH on chromosomes 13q, 17q, 17p, and 22q, and mutations of *TP53* and *KRAS* in tissue and peritoneal washes from patients with OvCa revealed the presence of tissue LOH in 38 % of peritoneal fluids. *TP53* mutations were in 21 % of tissue samples and all (100 %) were detectable in peritoneal fluid. In total, 57 % had genetic changes (all serous adenocarcinomas), and these changes were detectable in 62 % of matched peritoneal fluid. As a proof of principle, this has implications for OvCa early detection [14]. *TP53* mutations occur in up to 81 % of OvCas, and plasma *TP53* exon 5–8 mutations were detected in 16.7 % of preoperative patients [15]. Thus, genetic alterations from OvCa are concentrated more in peritoneal than circulating fluids.

13.4.4 Circulating OvCa Noncoding RNA Biomarkers

13.4.4.1 Deregulated miRNAs in OvCa Tissues and Cell Lines

MiRNA deregulation is associated with OvCa. In tissues, and also in body fluids, the aberrant expression of let-7 and miR-200 family members appears to be associated with various types of OvCa. Several miRNAs, possibly with tumor suppressor functions, are downregulated in OvCa tissues and cell lines. These include let-7d, miR-125b-1, miR-127, miR-140, miR-145, and miR-199a. Specifically, in serous OvCa, let-7b, miR-10b, miR-26a, miR-29a, miR-99a, miR-100, miR-125a, miR-125b, miR-143, miR-145, miR-199a, and miR-214 are downregulated. Similarly, a number of possible oncomirs are upregulated in

OvCa samples, and some are subtype associated. OvCa subtype-specific upregulated miRNAs include miR-30a-5p and miR-30a-3p in clear cell OvCa and miR-192 and miR-194 in mucinous OvCa, while serous OvCa is associated with increases in miR-16, miR-20a, miR-21, miR-23a, miR-23b, miR-27, miR-93, miR-141, miR-200a, miR-200b, and miR-200c. Some miRNAs deregulated in epithelial OvCa tissue samples also appear to correlate with various prognostic parameters such as DFS, PFS, Recurrence-FS, and OS. Those associated with good prognosis include miR-100, miR-150, miR-187, miR-200a, miR-200c, miR-335, miR-410, miR-645, and the ratio of miR-221/miR-222, while deregulated miR-21, miR-25, miR-29, miR-203, and miR-221 confer poor prognosis.

Several OvCa-associated miRNAs have defined functions in tumor initiation and progression and serve as therapeutic targets as well. Some miRNAs are implicated in EMT, cancer stem cell maintenance, angiogenesis, and extracellular matrix remodeling in OvCa. As examples, the tumor suppressor miR-138 represses genes involved in EMT and hence is downregulated in metastatic OvCa cells. Similarly, miR-125a inhibits EMT through AT-rich interactive domain 3B (*ARID3B*). EGFR signaling through the ETS family of transcription factor (*PEA3*) represses miR-125a expression in metastatic OvCa. MiR-200a and miR-200c are also downregulated in CD117-positive/CD44-positive OvCa stem cells compared to CD117-negative/CD44-negative cells. Increased expression of these miRNAs decreased ZEB1 and vimentin levels in association with E-cadherin expression, which impaired migration, invasion, and colony formation. MiR-125b, miR-145, and miR-199a target HIF-1α and VEGF to suppress tumor angiogenesis, and all are deregulated in OvCa. Finally, miR-335 is downregulated in OvCa, because it inhibits metastasis by targeting the glycoprotein tenascin C (TNC) that is a negative regulator of cell–extracellular matrix interaction.

13.4.4.2 Circulating OvCa miRNA Biomarkers

Circulating miRNA deregulation with diagnostic and prognostic potential are demonstrated in samples from OvCa patients. While mostly not validated, their potential role in the clinic is expected and should make a difference in the diagnosis and management of this disease.

In 2008, Taylor et al. first reported on differential levels of exosomal miRNA between women with OvCa and those with benign ovarian adenomas [16]. They selected eight known OvCa-associated miRNAs (miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, and miR-214) and demonstrated that these were significantly enriched in exosomes from cancer patients compared to controls. Of interest, these circulating exosomal miRNA levels were not correlated with tumor stage or grade, suggesting their potential as early OvCa detection biomarkers. The elevated levels of the miR-200 family members (miR-200a, miR-200b, and miR-200c) in sera from women with serous OvCa were confirmed. Resnick et al. reported a similar study, whereby 21 differentially expressed miRNAs in OvCa tissue samples were selected a priori, and tested on serum

samples from patients and controls [17]. Of the 21 miRNAs, five (miR21, miR-29a, miR-92, miR-93, and miR-126) were elevated, while the levels of three (miR-99a, miR-127, and miR-155) were decreased in sera from women with OvCa compared to healthy controls. Using whole blood samples from patients and controls, eight miRNAs (miR-16, miR-29a, miR-30c-1, miR-106b, miR-155, miR-146a, miR-191, and miR-383) were demonstrated to be deregulated in cancer patients, and this finding was consistent with those in OvCa tissue samples. miR-30c-1-3p was upregulated, while downregulated were miR-181a-3p, miR-342-3p, and miR-450b-5p [18]. A microarray profiling of sera, ascites, and tissue samples from women with serous OvCa uncovered four miRNAs (let-7b, miR26a, miR-132, and miR-145) that were significantly decreased in sera from patients compared to controls [19]. Plasma let-7f and miR-205 have also been identified as early detection biomarkers (stage I) of OvCa [20].

Endometriosis is a precursor lesion to some types of OvCa. The ability to differentiate endometriosis from OvCa is clinically relevant. The work by Suryawanski et al. sheds some light on the potential of miRNA in this regard [21]. Global profiling of circulating miRNA in women with endometriosis-associated OvCa enabled the identification of miR-16, miR-191, and miR-195 to be elevated in women with endometriosis. This miRNA signature had a detection sensitivity of 88 % and specificity of 60 %. Three other miRNAs (miR-16, miR-21, and miR-191) were able to differentiate women with endometriosis-associated OvCa from healthy women at a sensitivity of 86 % and specificity of 85 %. Importantly, miR-21, miR-362-5p, and miR-1274a performed at a sensitivity of 57 % and a specificity of 91 % in separating between women with endometriosis and those with endometriosis-associated OvCa. Moreover, miR-21, miR-191, and miR-1975 could stratify women with sOvCa and endometriosis-associated OvCa at a sensitivity of 86 % and specificity of 79 %. A separate set of miRNAs (miR-362-5p, miR-628-3p, and miR-1915) performed at a sensitivity of 90 % and specificity of 73 % in differentiating women with endometriosis from those with sOvCa. Finally, for separating between women with sOvCa and healthy controls, miR-16, miR-191, and miR-4284 achieved a sensitivity and specificity of 90 % and 55 %, respectively.

Several circulating miRNAs that are deregulated in OvCa have been associated with clinical outcomes. Decreases in let-7f levels correlate with poor prognosis, and elevated miR-221 in sera from women with eOvCa is associated with FIGO stage and tumor grade. In multivariate analysis, high circulating miR-221 was an independent predictor of poor prognosis [22]. Circulating levels of miR-92 correlate with lymph node metastasis and clinical stage of OvCa, while advanced FIGO stage, high tumor grade, and poor OS were associated with elevated miR-21 [23]. Five preoperative plasma miRNAs significantly predicted shorter OS. However, after multiple testing, only miR-1290 emerged as a robust prognostic biomarker, with AUROCC of 0.87, $p = 0.05$ [24].

13.4.5 Circulating OvCa Proteomic Biomarkers

Proteomic approaches have made important contributions in the identification of biomarkers for OvCa management. Still remaining as an important issue is the discovery of screening biomarkers. Because of the low disease prevalence, a sensitivity of >75 % and a specificity of >99.6 % are required to achieve an acceptable PPV of 10 %. Thus, there have been several proteomic biomarker discovery research efforts to meet these demanding performances.

13.4.5.1 Serum Proteomic Spectral Peaks as OvCa Biomarkers

Several discovery studies have explored the use of serum *m*/*z* spectral peak signatures for OvCa detection. The seminal work of Petricoin et al. is noteworthy [25]. This group used serum proteomic SELDI TOF MS spectra applied to an iterative search algorithm to identify a discriminatory pattern for cancer detection. This "cluster -proteomic -pattern" biomarker achieved a sensitivity of 100 %, a specificity of 95 %, and a PPV of 94 % for OvCa. Since then, several groups have applied proteomic spectral patterns as cancer biomarkers.

Four peptide peaks at *m*/*z* 6195, 6311, 6366, and 11498 achieved a sensitivity of 87 % and a specificity of 95 % in a validation sample set. A peak at *m*/*z* 4475 only appeared after chemotherapy [26]. Lin et al. observed four peaks (*m*/*z* 5147.06, 6190.48, 11522.6, 11537.7 Da) in plasma from cancer patients but not controls, and 2 peaks (5295.5, 8780.48) in controls but not in cancer patients [27]. The sensitivity and specificity of these peaks were 96.3 % and 100 % for OvCa. Wang et al. also observed seven discriminatory peaks at *m*/*z* 3940, 4099, 4144, 4479, and 5488 (upregulated) and 8588 and 13783 (downregulated) in advanced stage OvCa that had sensitivity of 93.94 % and specificity of 93.55 % [28]. These biomarkers also detected 90 % of early stage OvCas. Of 98 peaks that discriminated malignant from benign tumors, 46 were finally identified from average linkage clustering to be more relevant [29]. Similarly, in women with normal CA125 levels, 25 peaks discriminated malignant from the benign group [30]. Mass spectrometric peaks of sera could differentiate stage I/II disease from controls at a sensitivity of 80% with AUROCC of 0.82, and stage III/IV at a sensitivity of 93 % and AUROCC of 0.92. Note that the majority of these studies involved small samples sizes and hence are only proof-of-concept discovery findings.

13.4.5.2 Serum OvCa Proteomic Biomarkers

Proteomics approaches have enabled the identification of several proteins and peptides with differential levels in circulation of women with OvCa. Among these numerous proteins are haptoglobin, apolipoprotein A1, transthyretin, amyloid

AI, transferrin, β-hemoglobin, and β2-microglobulin that show potential as OvCa biomarkers.

Serum amyloid A1 and hemoglobin are among the early protein biomarkers identified by proteomic studies for OvCa. Moshkovskii et al. observed invariant peaks at *m/z* 11.7 and 11.5 kDa that were present in 55.6 % of OvCa but at low intensities in only 5.8 % of controls [31]. These peaks were identified as amyloid A1 (11.68 kDa) and its N-terminal arginine-truncated form (11.52 kDa). Woong-Shick et al. also identified two peaks with significant differential expression between cancer and controls as hemoglobin-α (15.1 kDa) and hemoglobin-β (15.8 kDa) chains [32]. The sensitivity for intact hemoglobin was 77 % in sera from cancer compared to controls.

Several investigators have demonstrated the clinical performance of serum apolipoprotein A1 in OvCa. Kozak et al. used SELDI TOF MS to identify 14 differentially expressed peaks categorized into three protein panels designated as screening panel (five candidates), validation panel I (five candidates), and validation panel II (four candidates) [33]. Independently, they all performed very well in differentiating between benign and malignant ovarian neoplasia (AUROCC of $\geq$0.90). The three panels correctly classified 93 % of blinded samples comprising of various ovarian tumors and normal patient samples. In a follow-up study, 5 of the original 14 peaks were identified as transthyretin (TT, 2 peaks with *m/z* 13.9 kDa and 12.9 kDa), β-hemoglobin (Hb, *m/z* 15.9 kDa), apolipoprotein A1 (Apo-A1, m/z 28 kDa), and transferrin (TF, m/z 79 kDa) [34]. These biomarkers improved early-stage OvCa detection with AUROCC of 0.933 (compared to 0.833 for CA125). However, CA125 incorporation increased the performance to 0.959. For mucinous type OvCa, the five biomarkers were more discriminatory than CA125 alone (AUROCC of 0.959 vs. 0.613), and CA125 incorporation failed to improve detection of mucinous type OvCa. Nosov et al. extended this study to include the detection of serous and endometrioid type OvCa by analysis of Apo-A1, TT, TF, and CA125 levels [35]. This study, which included 358 serum samples from patients with benign adnexal masses, early and late stage OvCa, as well as healthy controls achieved a sensitivity of 96 % for OvCa detection (a sensitivity of 98 % for detection of early-stage endometrioid type cancer). A multicenter case–control study to validate serum biomarkers focused on 3 proteins (Apo-A1, TT, and cleavage fragment of inter-alpha trypsin inhibitor heavy chain 4) for which immunoassays were available [36]. Independent cross-validation for detection of early-stage OvCa was undertaken. These biomarkers improved detection of OvCa by CA125. The 4-biomarker combination had a sensitivity of 74 % at a fixed specificity of 97 %. Clarke et al. examined a panel of seven markers (apolipoprotein A1, truncated transthyretin, transferrin, hepcidin, b-2-microglobulin, connective tissue activating protein III, and inter-alpha-trypsin inhibitor heavy-chain 4) for OvCa detection [37]. However, in a training set, only three of these (Apo-A1, TT, and CTAPIII) performed the best with a sensitivity of 54 % at a specificity of 98 %, while CA125 in this series had a sensitivity of 68 %, and the combination increased the sensitivity to 88 %. In a validation assay, 84 % sensitivity was achieved at the

set specificity of 98 %. This seven panel biomarker, however, failed to improve sensitivity beyond CA125 alone for preclinical detection of OvCa [38].

Haptoglobin 1 (HAP1) is another potential serum biomarker for OvCa. The work by Ye et al. using SELDI TOF MS and LC MS/MS identified a peak differentially expressed at a significant level between cancer and controls as alpha chain of haptoglobin [39]. This alone performed at a sensitivity of 64 % and specificity of 90 %, and together with CA125, the panel had a high sensitivity of 91 % and specificity of 95 % for detection of OvCa. Six peaks significantly upregulated in all groups of OvCa patients were identified as isoforms of haptoglobin 1 precursor [40]. This group subsequently identified a downregulated protein, transferrin, as well as the previously upregulated HAP1 in grade 3 OvCas [41]. The changes were also detected in peritoneal fluids from patients. Haptoglobin levels decreased (transferrin remained unchanged) following six cycles of taxol/carboplatin chemotherapy.

Several other potential serum proteins are uncovered for OvCa. Havrilesky et al. evaluated a panel of 9 biomarkers (HE4, glycodelin, MMP7, SLPI, Plau-R, MUC1, Inhibin A, PAI-1, and CA125) for early OvCa detection and 4 biomarkers (HE4, Glycodelin, MMP7, and CA125) for recurrence monitoring of OvCa [42]. For OvCa detection, the highest sensitivity and specificity achieved with various biomarker combinations were 80.5 % and 96.5 % for early-stage disease and 89.2 % and 97.2 % for late-stage disease. Recurrence was predicted in 100 % of the women with recurrent disease (compared to 96 % for CA125 alone). At least one biomarker was elevated earlier (6–69 weeks) than CA125, and before clinical detection of recurrence in 52 % of the patients. Of 6 proteins identified in another study, 2 (CCL18 and CXCL1) were selected by multivariate predictive model and validated [43]. Immunoassays were developed and used to screen 535 serum specimens comprising of people with OvCa, benign pelvic masses, other non-gynecologic cancers, and healthy controls. As a panel, the two biomarkers had a sensitivity of 92 % and a specificity of 97 % for OvCa detection. In combination with CA125, the sensitivity improved to 99% with healthy women, and 94 % for women with benign pelvic masses as controls, at a specificity of 92 %. Sera collected several months before cancer diagnosis from 295 women and 585 controls were profiled by MALDI MS for early detection biomarkers [44]. Two peaks, CTAPIII and platelet factor 4 (PF4), in combination with CA125 were discriminatory between cancer and controls months before cancer diagnosis, and this was earlier than predictions by CA125 alone.

Other multi-analyte serum biomarkers have proven clinically useful for OvCa. Zhang et al. tested the utility of 4-biomarker panel consisting of CA125II, CA72-4, CA15-3, and M-CSF in an artificial neural network-derived composite index for detection of early-stage OvCa [45]. The panel performed much better than CA125II alone in the detection of early-stage OvCa with a sensitivity of 71 % at a set specificity of 98 %. Hogdall et al. similarly examined 7-marker panel for utility in triaging women with pelvic masses for subsequent evaluation [46]. In multivariate logistic regression analysis, CA125, age, and the 7-proteomic biomarkers had independent predictive abilities of OvCa. The combination of these metrics (the

7-marker panel, CA125, and age) constituted the Danish Index (DK-Index). For the detection of EOC, the DK-Index had a sensitivity of 95 % and a specificity of 81 %, which was much superior to CA125 alone. Phase II multi-analyte biomarker (CA125, CRP, serum amyloid A, IL-6, and IL-8) trial for OvCa diagnosis included sera from 150 cases and 212 controls, and was validated in an independent cohort of 183 women. The 5-panel biomarker performance was significantly greater than CA125 alone in the validation cohort, as well as for stage I/II disease. Sensitivity and specificity were 94.1 % and 91.3 % for the validation cohort, and 92.3 % and 91.3 % for early-stage disease [47]. In a subsequent large-scale study, the 5 serum biomarkers were tested on sera from 222 women with EOC, 223 with benign disease, 53 with borderline OvCa, and 244 healthy controls [48]. The biomarkers were significantly elevated in sera from OvCa women compared to all the control groups. Assay performance was significantly much better than CA125 for discriminating borderline EOC from benign and healthy controls (AUROCC was 0.884 vs. 0.843 for CA125). At a specificity of 95%, the in vitro diagnostic multivariate index assay (IVDMIA) achieved a sensitivity of 69.5 % (compared to 62.5 % for CA125 alone).

13.4.6 Serum Protein Biomarkers in Clinical Use for OvCa Management

Numerous serum protein biomarkers have shown promise at the research level. Currently, however, only CA125 and HE4 are FDA approved for monitoring OvCa progression.

13.4.6.1 Serum CA125 as OvCa Biomarker

Bast and colleagues identified CA125 in 1981, when developing the monoclonal antibody OC125 against the serous OvCa-derived cell line, OVCA 433 [49]. It is a high molecular weight glycoprotein encoded by *MUC16*. The protein comprises of a tandem repeat of 156 amino acids at its N-terminal, a phosphorylation site at the C-terminal, and a possible transmembrane region.

The commercial immunoassay originally marketed in 1983 used the OC125 antibody. A second iteration of the test included another antibody, M11, with distinct epitope from OC125. The FDA recommends CA125 for limited intended use in the management of OvCa. However, studies show it has value in screening, differential diagnosis of a pelvic mass, treatment response and recurrence monitoring, as well as prognostic prediction.

While useful, CA125 alone faces numerous confounding factors. CA125 levels are influenced by factors such as age and race. Aging is associated with decreasing levels, and race-associated variations are observed in women after menopause. A recorded 20–50 % variation is recorded, with African-American women having

lower concentrations than white women. Moreover, menstrual cycle affects serum levels, and levels increase in 1–2 % of healthy people, in 5 % of women with other gynecological conditions, and in up to 28 % of non-gynecological malignancies.

The CA125 assay is affected by analytical variables such as sample handling. Samples for CA125 analysis are therefore recommended to be assayed as soon as serum is separated; otherwise, they can be refrigerated at 4 °C for up to 5 days or frozen at −20 °C for short term (2 weeks to 3 months) or deep frozen at −70 °C for long term storage.

Serum CA125 as OvCa Screening Biomarker

Eighty percent of women with EOC have elevated serum CA125, with the following stage distribution: 50–60 % of stage I, 90 % of stage II, and over 90 % of stage III/IV women. But CA125 has not achieved the desired accuracy or evidence of reduced mortality to be a stand-alone screening test in asymptomatic women. However, women with histories of hereditary OvCa, who have a 40% risk of developing this disease over their lifetime, are recommended by the NIH consensus Development Panel to have annual CA125 screening coupled with physical pelvic examination and transvaginal ultrasound for early cancer detection. For effective screening, an OvCa biomarker must have a very high sensitivity of > 75 % and a specificity of 99.7 % to attain the acceptable PPV of 10 % among women over 50 years who have a disease prevalence of 40/100,000. Because CA125 alone, and for that matter many biomarkers, cannot reach such high accuracy, several strategies have been adopted by many investigators to attain this performance. These strategies include longitudinal measurement of CA125 (ROCA), use of CA125 with other biomarkers in panels or IVDMIAs, and the original CA125 and transvaginal ultrasound.

Serum CA125 as a Biomarker for Differential Diagnosis of a Pelvic Mass

Attempts have been made to use CA125 to estimate the risk for OvCa. Indeed, in postmenopausal women, CA125 levels above 95kU/L can discriminate women with malignant ovarian tumors from those with benign tumors at a positive predictive value of 95 %. For clinical utility, CA125 is currently used in RMI, OVA, and ROMA algorithms for such predictions (see sect. 13.4.6.3).

Serum CA125 as OvCa Treatment Monitoring Biomarker

In women on chemotherapy following surgery, CA125 is used to monitor treatment outcomes. A declining CA125 levels indicates response to treatment, and the reverse is true. Guidelines are needed for consistent clinical use. Thus, the Gynecologic Cancer Intergroup (GCIG), which is made up of 13 international groups engaged in gynecologic cancer clinical trials, provided some leadership in this direction. For recurrence prediction, CA125 levels must be 50 % or higher from pretreatment levels, and this should be maintained for at least 28 days [50, 51]. Also pretreatment CA125 levels must be at least twice the upper limit of the reference range for this prediction to be valid (the limitation is that this excludes some women who do not satisfy such stringent criteria). Other needed requirements are that the

samples be taken within 2 weeks before commencement of therapy, then 2–4 weeks during treatment, and 2–3 weeks on follow-up monitoring. In salvage therapy, a doubling of CA125 predicted disease progression and failed therapy in over 90 % of cases [52]. Clinical trial for such use is being undertaken at the UK medical research council (OV05 clinical trial of CA125 vs. clinical parameters of recurrent prediction).

Serum CA125 as OvCa Recurrence Monitoring Biomarker
Early detection of recurrences enables prompt and appropriate interventions that provide some modest improvement in survival. Additionally, early detection offers ample time for the right chemotherapy to be identified for palliative treatment. Elevated CA125 may precede clinical and imaging findings of OvCa relapse for about 2–6 months median duration. Thus, the GCIG advocates the use of postoperative CA125 measurements to monitor recurrences. The criteria set is that for patients with elevated pretreatment CA125 levels that normalized on therapy, progressive disease is when CA125 concentration becomes equal to, or higher than twice the upper limit of normal on two occasions. For women with persistently elevated pretreatment CA125 that fail to normalize, progressive disease is defined by CA125 levels within the reference range, or above twice the nadir value on two occasions. Samples for the two measurements must be taken at least one week apart.

Serum CA125 as OvCa Prognostic Biomarker
Several studies indicate that both pretreatment and postoperative CA125 levels have prognostic value in primary treatment of OvCa. Declining levels after surgery and chemotherapy are independent prognostic factors. Preoperative levels >65kU/L are associated with significantly lower 5-year survival, and these patients have a 6.37-fold risk of death compared to those with lower levels [53, 54]. Additionally, the circulating half-life of CA125 may predict disease outcome. Half-life less than 20 days is associated with significantly longer survival than those with prolonged half-life greater than 20 days (28 vs. 1 months). Normal levels obtained after three cycles of chemotherapy confer better survival [52, 55].

13.4.6.2 Serum HE4 as OvCa Biomarker

Human epididymis protein 4 (HE4) is a 25kDa-secreted glycoprotein encoded by *WFDC2*. Identified in 1991 by Kirchhoff et al., it belongs to the “4-disulfide core” family of proteinase inhibitor family [56]. Initial expression analysis revealed its presence in the distal regions of the epithelial cells of the epididymal ducts; hence, it was postulated to have a role in sperm maturation. Work by Schummer et al. using cDNA CGH, however, revealed overexpression in OvCa compared to normal ovarian tissue, and this finding was corroborated by SAGE expression analysis [57, 58]. It is involved in OvCa cell migration, adhesion, growth, proliferation, and overall tumor progression.

Cancers of the lungs, breast, pancreas, gastrointestinal tract, kidneys, endometrium, and bladder also express HE4. However, the highest levels are in ovarian and endometrial cancers. The frequencies of expression are related to the histologic type of OvCa, being present in 100 % of endometrioid cancers, 93 % of serous tumors, and 50 % of clear cell tumors but not in mucinous tumors. Several normal tissues including those of the respiratory tract, genital tract, breast, distal renal tubules, colon, salivary glands, fallopian tubes, epididymis, and vas deferens also express HE4. The highest levels are in the trachea and salivary glands. However, these expression levels are still much lower than those from ovarian and endometrial cancers [59]. Hellstrom et al. developed monoclonal antibodies for assaying HE4 in serum samples [60]. Used in a blinded study, this initial assay demonstrated performances comparable to CA125, but HE4 elevation in other benign gynecologic conditions was much less infrequent.

The clinical utility of HE4 in OvCa has been determined in meta-analytical studies. One systematic review and meta-analysis of studies between 1990 and 2011 on HE4 included nine studies involving 1807 women [61]. The pooled sensitivity and specificity were 83 % and 90 %, respectively, for OvCa detection with healthy women as controls, with a SAUROCC of 0.9271. With benign gynecologic conditions as controls, HE4 had a pooled sensitivity, specificity, and SAUROCC of 74 %, 90 %, and 0.8853, respectively. Another meta-analysis of the diagnostic accuracy in differentiating between OvCa and benign gynecologic diseases included 11 studies involving 3395 patients [62]. In this expanded study, the pooled sensitivity, specificity, PLR, and NLR were 74 %, 87 %, 0.804, and 0.27, respectively. When combined with CA125, the sensitivity was enhanced at a compromised specificity. Thus, HE4 serves as a biomarker with potential for screening, prognostication, recurrent and therapy monitoring, and importantly, it is included in an algorithm (ROMA) for discriminating between benign and malignant pelvic masses. Caution is however warranted in the interpretation of HE4 results, because levels are altered by:

- Non-ovarian malignancies—elevated especially in lung cancer.
- Age: levels are significantly increased in postmenopausal compared to premenopausal women.
- Renal diseases: renal failure or fibrosis increases circulating levels.
- Pregnancy: levels are significantly decreased.
- Menarche: late menarche is significantly associated with high levels.
- Smoking significantly elevates the levels.

Serum HE4 as OvCa Screening Biomarker

The heterogeneous nature of OvCa (types I and II) with their different molecular pathologies indicates that no single biomarker is likely to achieve the sensitivity and specificity required for screening of the designated population. Consequently, HE4 is incorporated in panels for assessing their utility in screening. It should be noted that HE4 is expressed in 32 % of OvCa cells that do not express CA125. Additionally, in over 50 % of OvCas patients with negative serum CA125, HE4 levels are elevated. Multiple studies also show different performances for these two

biomarkers with regard to sensitivity (CA125 higher) and specificity (HE4 higher). All these observations indicate that complementary utility of both biomarkers is worthwhile.

Serum HE4 as OvCa Prognostic Biomarker

Multiple studies suggest possible prognostic utility of HE4 alone or in combination with CA125 or other biomarkers in algorithms. For example, elevated HE4 and ROMA are independent predictors of shorter survival, progression-free survival, and disease-free survival. Similarly, ROMA, elevated HE4, and CA125 all correlate with advance age, stage, lymph node involvement, presence of ascites, and suboptimal cytoreduction. Elevated HE4 levels are associated with FIGO stage, grade, preoperative CA125 levels, and residual tumor volume. Tumor aggression, overall survival, and poor prognosis are associated with elevated HE4. In the OVCAD study, elevated HE4 levels were associated with poor surgical outcome [63]. HE4 was found to be a better predictor of optimal cytoreduction, with a sensitivity of 100 % and specificity of 89.5 % when a cutoff value of $\leq$262 pM was used.

Serum HE4 as OvCa Recurrence Monitoring Biomarker

Compared to CA125, MMP7, and mesothelin, in advanced stage OvCa following surgery and chemotherapy, HE4 is elevated prior to recurrence at lead -time of 4.5 months. In some recurrent patients with increasing CA125, HE4 increases before CA125. A cutoff value of 70 pmol/L of HE4 had a sensitivity of 74 and specificity of 100 % in recurrence detection. Incorporation of CA125 marginally increased the detection rate to 76 %. Both CA125 and HE4 levels increase in response to peritoneal spread, in both patients with small implants and those with imaging evidence of macronodular implants and omental thickening. Statistical significance was achieved for HE4 (but not CA125) in detecting small and macronodular implants. Elevated HE4 levels are also associated with lymph node involvement.

Serum HE4 as a Biomarker for Differential Diagnosis of a Pelvic Mass

Biomarkers that augment the diagnostic assessment of a pelvic mass are needed. HE4 is less frequently elevated in benign gynecologic conditions than CA125 (only 8 % compared to 29 %), especially in premenopausal women. For example, in endometriosis, CA125 is elevated in up to 67 % of patients compared to just 3 % for HE4. Diagnostic accuracy of HE4 for differentiating malignant OvCa from benign disease in evidence-based medicine had an overall pooled sensitivity of 74 % and specificity of 87 % [62]. The combination of CA125 and HE4 performs better than either alone, and is much better at discriminating pelvic masses than other panels. The use of CA125, HE4, and age achieved an AUROCC of 0.797. This is the basis for the use of these markers in FDA-approved algorithms for delineation of the pathologic nature of pelvic masses.

13.4.6.3 Biomarkers Used in Algorithms for OvCa Risk Prediction

In gynecologic practice, many women often present with pelvic masses, of which OvCa is not the most frequent diagnosis. Triaging these women for possible OvCa diagnosis, and hence referral to gynecologic oncologists where optimal management is offered is critical to disease outcome. Effective cytoreduction and improved chemotherapy by these oncologists have contributed significantly to the overall increase in survival over the past few decades, with 5-year survival change from 37 % in 1974–1976 to 46 % in 1999–2005.

Traditionally, a pelvic mass is evaluated by physical examination coupled with transvaginal ultrasound. Physical examination cannot diagnose cancer, and ultrasound has less than average performance with a sensitivity of 75 % but a dismal positive predictive value of 3–5 %. Computerized tomographic scans and MRI are good but expensive. PET/CT has the best performance in diagnosing pelvic masses but is cost-prohibitive as well. Additionally, imaging modalities are not accessible in many clinical settings, especially in resource-poor communities. Thus, the Society of Gynecologic Oncologists and the American College of Oncology and Gynecology recommend the use of CA125, presence of ascites, local or distant spread, findings on physical examination, as well as family history as a criteria for referral to gynecologic oncologists. Admittedly, these findings are often more associated with late-stage disease, and will not enable early detection of OvCa. The need for noninvasive multi-panel biomarkers for OvCa has been pursued vigorously resulting in some encouraging tests and algorithms being developed. The FDA has thus approved OVA and ROMA for determination of risk of OvCa in women with pelvic masses.

Risk of Malignant Index as OvCa Risk Prediction Biomarker
Described in 1990 by Jacobs and colleagues, the first multi-analyte diagnostic index widely used (especially in Europe) for assessing pelvic masses is the "risk of malignancy index" (RMI). This index includes incorporating serum levels of CA125 with ultrasound scores and menopausal status to compute the risk of a pelvic mass being malignant or benign. It is a very simple cost-effective algorithm, involving simple multiplication of the three scores: CA125 x menopausal status (one for premenopausal and three for postmenopausal women) x ultrasound scores (0, 1, or 3 depending on the features). This algorithm has an overall sensitivity of 85 % and specificity of 97 % in determining the benign or malignant nature of a pelvic mass. Multiple trials however indicate the accepted performance has a sensitivity range of 71–88 % and a specificity of 74.3–97 %. A recent large Danish study of 1159 women presenting with pelvic masses at a tertiary institution achieved sensitivity as high as 92 % at a specificity of 82 %. The NPV and PPV were remarkable at 97 % and 62 %, respectively [64]. A systematic review by Geomini et al. using a bivariate meta-analysis to estimate pooled performance indicated RMI has a pooled sensitivity of 78% and specificity of 87 %, which was much superior to other models tested in this study [65].

OVA as OvCa Risk Prediction Biomarker

In September 2009, the FDA approved the first serum-based multi-analyte biomarker test for the differential diagnosis of a pelvic mass in women, specifically to differentiate between OvCa and benign adnexal masses. This assay involves the measurement of five serum protein biomarkers that were identified through SELDI TOF MS proteomic profiling. Following biomarker stratification, validation, and the development of immunoassay, the following biomarkers were selected: CA125, apoliporotein A1, transferrin, transthyretin, and β2-microglobulin. The serum levels of these biomarkers in combination with imaging and menopausal status are used to generate a multivariate index risk score (between 1 and 10) for OvCa. Cutoff values for premenopausal women is 5 and for postmenopausal women is 4.4. Scores above this indicate elevated risk for OvCa in the presence of a pelvic mass, and these women require referral to gynecologic oncologists for expert investigation and treatment.

The performance of OVA1 in the differential diagnosis of a pelvic mass has undergone clinical trials [38]. In comparison to CA125 alone, OVA1 was superior with a sensitivity of 94 %, specificity of 35 %, and NPV of 93 %. The sensitivity and NPV were higher than CA125, but there is a trade-off in specificity and PPV. Similar performances of OVA1 vs. normal physician assessment of pelvic masses achieved a sensitivity of 96 %, specificity of 35 %, and NPV of 95 %. Also OVA1 vs. gynecologist oncologist assessment performed at a sensitivity of 99 %, specificity of 26 %, and NPV of 98 %. OVA2 is being developed to improve performance, especially the dismal specificity of OVA1. This assay includes 9 serum biomarkers, namely CA125, HE4, IL-2Rα, α-1-antitrypsin, CRP, YKL-40, cellular fibronectin, CA72-4, and prostasin. Preliminary assessment achieved a sensitivity of 88.9 % (compared to 63.4 % for CA125 in this study) and specificity of 69.1 % (much improved from OVA1). The increased specificity is likely due to the incorporation of HE4 that has such high specificity for OvCa. The assay currently uses five top performing targets, namely CA125II, ApoA1, TRF, FSH, and HE4 to generate a dichotomized risk prediction score (<5.0 as low risk and ≥ 5.0 as high risk). Vermillion intends to submit this to the FDA for 510(k) clearance.

Risk of OvCa Algorithm as OvCa Risk Prediction Biomarker

The "risk of OvCa algorithm" (ROCA) is a computerized algorithm that incorporates longitudinal measurements of CA125 and a woman's age to generate a predictive risk index for OvCa. Based on the output, the individual risk could be low, intermediate, or high. This algorithm helps the triaging of women for referral to centers where optimal OvCa investigation, early detection, and management can occur. In general, increasing CA125 levels in association with advancing age is suggestive of an elevated OvCa risk. On the contrary, a woman can have elevated CA125 levels above the designated cutoff value of 35 u/ml and yet be considered low risk because this elevated level remained unchanged over several years. This algorithm has a sensitivity of 86 % and a specificity of 98 % for OvCa risk prediction.

Risk of Ovarian Malignancy Algorithm as OvCa Risk Prediction Biomarker
A second FDA-approved test for OvCa in September 2009 is the risk of ovarian malignancy algorithm (ROMA). ROMA uses an algorithm that combines CA125, HE4, and menopausal status to establish possible risk of OvCa. These variables are first used to compute different predictive indices (PI) for premenopausal and postmenopausal women, which are then used to calculate the predictive probability (PP) for the risk of OvCa in a woman presenting with a pelvic mass:

- PI for premenopausal women $= -12.0 + 2.38 \times \text{In(HE4)} + 0.0626 \times \text{In(CA125)}$.
- PI for postmenopausal women $= -8.00 + 1.04 \times \text{In(HE4)} + 0.732 \times \text{In(CA125)}$.
- $\text{PP} = \text{EXP(PI)}/[1 + \text{EXP(PI)}] \times 100$ (for percentage).

Fujirebio Diagnostic In (FDI) and Abbott commercialize this product, but each uses a different cutoff value. For FDI, levels equal to or above 13.1 for premenopausal and 27.7 for postmenopausal women indicate elevated risk. The corresponding cutoff values used by Abbott for premenopausal and postmenopausal women are 7.4 and 25.3, respectively. The algorithm performs much better in premenopausal than postmenopausal women. Sensitivities of up to 92.3 % and specificity of 75.0 % for premenopausal women have been achieved. For postmenopausal women, sensitivity is 76.5 % and specificity is 74.8 %

Several scientific evidences validate the performance of ROMA. Moore et al. in a multicenter study of nine serum biomarkers in 233 women presenting with undiagnosed pelvic mass identified HE4 as the most sensitive biomarker within the panel [66]. When coupled with CA125, they provided the best classification accuracy with a sensitivity of 76.4 % and a specificity of 95 % for differentiating between malignant and benign pelvic masses. In 2009, Moore and colleagues further evaluated these biomarkers in a prospective study of 531 women [67]. These biomarkers in an algorithm with menopausal status correctly classified 93.8 % of the cancer patients as having high-risk scores for OvCa, at a specificity of 74.9 %. Other studies confirmed this assay as being invaluable in pelvic mass assessment [68–70]. Nolen et al. studied 750 patients, examining 65 biomarkers [68]. CA125 and HE4 were the best performers, and in a validation cohort, they achieved a sensitivity of 83 % and a specificity of 85 %. Huhtinen et al. used the two biomarkers to predict malignant ovarian masses from benign cysts at a sensitivity of 92.9 % and specificity of 95 % [69]. Holcomb et al. similarly achieved a sensitivity of 91.2 % but a slightly low specificity of 55.4 % [70]. ROMA may benefit from incorporation of imaging in the algorithm. Work by Macuks et al. where CA125, HE4, and menopausal status (as in ROMA) were combined with ultrasound scores, the AUROCC was as high as 0.939 for discriminating between benign and malignant pelvic masses [71].

13.4.6.4 Serum Mesothelin as OvCa Biomarker

Mesothelin is a glycoprotein of mesothelial cells of the pleura, pericardium, and peritoneum. OvCa cells overexpress it. Mesothelin contributes to OvCa cell migration and metastasis partly through ERK1/2, AKT, and JUNK-mediated regulation of MMP7. In postmenopausal women, expression is associated with advancing age and inversely with BMI [72]. Serum levels are potential diagnostic biomarkers of OvCa. A prognostic utility has also been demonstrated. High levels in women after optimal cytoreduction/debulking surgery or those with advanced stage disease are associated with poor overall survival [73]. The frequency of detection of mesothelin in urine is much higher (42 %) than in serum (12 %) in women with early-stage OvCa [74]. In a panel with CA125, the sensitivity for OvCa detection was enhanced [75].

13.4.7 Circulating OvCa Metabolomic Biomarkers

A few proof-of-concept studies have uncovered glycomic and metabolomic signatures for OvCa. In one study, at least 15 unique glycan biomarkers were demonstrated in all OvCa patients that were absent in normal individuals [76]. In another study, serum glycans were cleaved using solid-phase extraction and subjected to MALDI Fourier transformation MS [77]. Sixteen unique oligosaccharide MS signals were identified in OvCa samples. This glycomic assay achieved a sensitivity and specificity of 91.6% and 95.8% with an AUROCC of 0.954 for OvCa detection. Li et al. separated glycosylated serum proteins by gel electrophoresis and then analyzed for glycoprotein identification [78]. The work in OvCa cell lines indicated most glycosylated proteins were O-linked glycans. Four glycoproteins masses at 517, 370, 250, and 163 kDa were identified as two isoforms of apolipoprotein B-100 (517 and 370 kDa), fibronectin (250 kDa), and immunoglobulin A1 (163 kDa). Apolipoprotein B-100 was O-linked, but the other two were N-linked glycans that differed in form from those in sera from normal individuals (altered glycosylation in OvCa). A direct serum glycan analysis without protein identification was developed for biomarker discovery [79]. In this study, global glycan release was achieved with chemical (O-linked) and enzymatic (N-linked) treatment and isolated by solid-phase extraction before analysis by MALDI Fourier transform ion cyclotron resonance MS. Potential glycan biomarkers were identified for ovarian, breast, and prostate cancers.

1H-NMR spectroscopic analysis of serum correctly classified cancer from healthy premenopausal women and those with benign ovarian diseases at a sensitivity of 100 %. For postmenopausal women, the accuracy was 97.4 %. MS metabolic profile of tissue samples could differentiate invasive cancer from borderline tumors based on 51 metabolites. These metabolites correspond to metabolic pathways that control pyrimidine metabolism and also are prognostic indicators of OvCa.

13.4.8 Circulating OvCa Cells

Bone marrow disseminated tumor cells (DTCs) and CTCs have been evaluated in women with OvCa for their clinical utility in patient management. While an invasive sample acquisition process, DTCs have shown promise as prognostic biomarkers for OvCa. Circulating OvCa cells (COvCaCs) are also emerging as prognostic biomarkers and should replace DTCs with validation findings from the various studies being conducted.

DTCs in OvCa patients were initially reported in 1990 and confirmed 5 years later. Both these pioneering studies employed immunocytochemistry but with different antibodies. DTCs often remain dormant for a period of time following which they reenter the circulation to colonize preferred "soils" at distant sites. In view of this, the association of DTCs with COvCaCs in women has been explored. In 2002, Marth et al. in a pilot study demonstrated the association of DTCs with COvCaCs [80]. In this series, 9 patients with COvCaCs harbored DTCs, but 6 patients were positive for only DTCs (without detectable COvCaCs). The latter finding is not surprising because the controlling factors for DTC release, and hence their temporal presence in circulation are unknown. Consistently, in a large ($n = 122$) prospective study, the concordant rate of DTCs and COvCaCs was <60 %.

Following these studies, the prognostic role of DTC in OvCa has been demonstrated by a number of studies. Bone marrow aspirates prospectively collected from 108 newly diagnosed women with OvCa were characterized for DTCs using anti-cytokeratin antibody, A45-B/B3. Thirty percent of the patients were positive for DTCs. In multivariate analysis, a median 45-month follow-up indicated that the presence of DTC was significantly associated with decreased distant disease-free survival for all patients (RR of 13.8, $p < 0.0001$), as well as OS (RR of 2.3, $p = 0.01$). Banys et al. also demonstrated a significant association of DTC-positivity among 112 women with shorter DFS compared to those negative for DTCs (22 vs. 31 months, $p < 0.05$) [81].

Other studies have attested to the prognostic relevance of COvCaCs in regard to PFS, DFS, and OS. Fan et al. [82] found a significant reduction in DFS in patients with COvCaCs, and Aktas et al. [83] made similar observations in patients before surgery ($p = 0.0045$) and after chemotherapy ($p = 0.047$). In a large cohort (216) of women with OvCa participating in a phase III clinical trial, prospective evaluation of COvCaCs using the CELLSEARCH® system was conducted at baseline and at intervals of every 2 months [84]. COvCaCs > 2/7.5 ml of blood was associated with poor prognosis in this cohort. Of the 14 % with baseline COvCaCs >2/7.5 ml, univariate analysis was significantly associated with reduced PFS and OS (HR 1.6, $p = 0.057$), but multivariate analysis fell short of significance (HR 1.5, $p = 0.096$). In a similar study using the CELLSEARCH® system on women with recurrent OvCa on temsirolimus as part of a phase II trial, COvCaC enumeration failed to predict survival [85]. Obermayr et al. used global gene expression to identify novel COvCaC markers that were used in conjunction with EpCAM to isolate and characterize COvCaCs from 2016 women prior to treatment, and 6 months following platinum-based chemotherapy [86]. Many (~66 %) COvCaCs

were detected by overexpression of cyclophilin C (PPIC), with just a few by EpCAM expression. This should help explain the low recovery rate and discordant results using the CELLSEARCH® system. Baseline COvCaCs correlated with high CA125 and HE4 levels, ascites, and suboptimal surgical tumor removal. Women who were positive for COvCaCs posttherapy were older and resistant to chemotherapy, and this was associated with decreased DFS (HR 3.5, $p = 0.001$) and OS (HR 2.3, $p = 0.02$). Pearl et al. used a cell adhesion matrix-based, and functional cell enrichment and identification platform to isolate the invasive subpopulation of COvCaCs from 129 preoperative OvCa patients [87]. This approach enabled high COvCaC detection, at a sensitivity of 83 % and PPV of 97.3 % for this invasive cell subpopulation in patients of all stages, and a sensitivity of 41.2 % and PPV of 77.8 % for stage I/II disease patients. The presence of these cells significantly predicted DFS and OS much better than elevated CA125 levels.

13.4.9 Circulating OvCa Stem Cells

Ovarian and other solid tumors are currently been viewed as stem cell pathology, and hence intense efforts are in place to identify these cells, study their behavior, and therapeutically target them. In OvCa, stem cells are defined by the expression of specific genes and engagement of specific signaling pathways, in addition to their stemness features as other somatic stem cells. Additionally, these cells tend to be resistant to therapy and have efficient metastatic potential. The epithelial OvCa stem cell is defined by the expression of CD133, CD44, CD117/c-kit, CD24, and DEAD box polypeptide 4 (DDX4). Bapat et al. first reported on the presence of EOC stem cells [88]. These tumorigenic stem or progenitor clone was isolated from ascites fluid from a patient with advanced OvCa.

In view of their rarity, their presence in circulation is not well characterized. However, some progenitor cell populations in circulation may have relatedness to the real OvCa stem cell. Circulating endothelial cells, defined as expressing CD34 and CD133 but negative for CD45 were demonstrated in women with OvCa ($n = 14$), and their levels declined following chemoradiation or surgical therapy [89]. Another population of cells defined as CD34+/VEGFR+ and referred to as lymphatic and vascular endothelial progenitor cells were significantly elevated in circulation of women with EOC, and their levels correlated with lymph node metastasis [90].

13.5 Summary

- OvCa is the most lethal gynecologic cancer, although the 5-year survival has improved due to effective disease management.
- Early detection is associated with 5-year survival rate of >90 %.

- The need for early detection biomarkers has led to the search for novel biomarkers, including the development of IVDMIAs.
- Type I (less aggressive) and type II (aggressive) OvCa subtypes have different molecular pathology, presentation, and prognosis.
- A number of novel circulating biomarkers for OvCa, including epigenetic and genetic signatures, as well as novel proteomic discoveries and panel biomarkers have been uncovered.
- Circulating CA125 and HE4 are clinically useful OvCa biomarkers.
- Circulating OvCa cells are promising prognostic liquid biopsy biomarkers.

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Chapter 14
Endometriosis Biomarkers in Body Fluids

Key Topics

- Molecular pathology of endometriosis.
- Circulating endometriosis miRNA biomarkers.
- Circulating endometriosis proteomic biomarkers.
- Circulating endometriosis immunologic biomarkers.
- Endometriosis biomarkers in other body fluids.

Key Points

- Endometriosis is a major cause of morbidity in women in their reproductive age. It is also a major global economic burden on health care. Thus, noninvasive early detection biomarkers of endometriosis are needed to mitigate these situations.
- Although mostly benign, ~1 % of endometriosis will progress to malignancy mostly involving the ovaries. The lesions with malignant potential often demonstrate LOH, MSI, and mutations in members of key signaling pathways including *PTEN*, *TP53*, and *CTNNB1*.
- Emerging noninvasive biomarkers of endometriosis include miRNAs, immunologic mediators, angiogenic factors, and novel proteomic discoveries in serum and urine (also in peritoneal fluids).

14.1 Introduction

Endometriosis is a condition of implantation and growth of endometrial tissue outside the uterus. It is a debilitating disease that can in some instances (a slight elevated risk) progress to ovarian cancer. It is a morbid condition of women in their

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reproductive ages and is associated with subfertility, nulliparity, and their associated psychological effects. The ectopic endometrial tissue is associated with inflammation, which alters ovarian functions and endometrial quality necessary for good oocyte production and nidation. Between 5 and 10 % of women in their reproductive ages are living with this condition, and for those reporting with subfertility and/or pelvic pain, as many as 35–40 % are diagnosed with endometriosis. Endometriosis is a major cause of disability in women and a great global economic burden on health care. It is the third leading gynecologic-related hospitalization in the USA and accounts for 25 % of gynecologic surgeries in China.

While not well grounded, the putative risk factors for endometriosis include genetics, nulliparity, pelvic infections, inflammation, and any gynecologic pathology that precludes normal outflow of menstrual tissue. Population and monozygotic twin studies suggest a familial component to endometriosis. The risk is six times higher in women who have first-degree relatives of severe endometriosis. Linkage analysis implicates chromosomes 7 and 10, but the specific genes implicated in endometriosis are still a challenge to gynecologic oncologists for the fact that it remains an enigma.

There currently are no validated circulating biomarkers for the noninvasive detection of endometriosis. Thus, the diagnosis still relies on the gold standard of invasive laparoscopic visualization and acquisition of endometrial samples for histopathologic evaluation. Transvaginal ultrasound, CT, and MRI scans are useful adjuncts to the diagnostic work-up. It will therefore be of considerable economic importance to have noninvasive molecular signatures for the diagnosis and management of endometriosis, and these are being pursued. Ideally, such noninvasive tests should include validated accurate biomarkers in the peripheral circulation, urine, saliva, or menstrual fluid. This chapter considers all such possible biomarkers for endometriosis management.

14.2 Screening Recommendations for Endometriosis

There are unfortunately no recommended screening tests or guidelines for endometriosis despite its high incidence and prevalence among women of reproductive age and the economic and psychologic burden on society. A cost-effective noninvasive test should reverse this situation. Currently, only a diagnostic workflow is offered for symptomatic women. These include thorough clinical history, physical examination, possible therapeutic trial (especially in women presenting with pelvic pain), imaging and eventual laparoscopic pelvic visualization, and biopsy of lesions for histopathology.

14.3 Molecular Pathology of Endometriosis

Endometriosis is a complex multifaceted disease with yet unclear etiopathogenesis, possibly due to the myriads of etiologies and molecular pathologies. Thus, a network of communications between environmental, epigenetic, genetic, hormonal, and immunologic factors underly endometriosis pathogenesis. A hereditary component is also suggested because there is an increased risk of 5–7 % in women who have affected first-degree relatives.

Endometriosis is a disease of women in their reproductive age, afflicting 5–10 % of women in this age group. However, there is a disproportionate clustering among infertile women (30–40 %) and/or those who report with pelvic pain. Several treatment modalities are available, but surgery is often offered to remove the endometriotic tissues. Recurrences after surgery are common, being 30 % at 3 years and up to 50 % in 5 years after surgery.

Anatomically, endometriotic tissues are found on the ovaries, fallopian tubes, cervix, vagina, rectovaginal septum, pelvic peritoneum, uterosacral ligaments, bladder, rectum, and the intestines (Fig. 14.1). They could, however, be found anywhere in the body as in very rare occasions ectopic endometrial tissues have been detected in the pleura and brain.

The American Society for Reproductive Medicine stages endometriosis based on the number, location, and depth of invasion of lesions, as well as the presence of

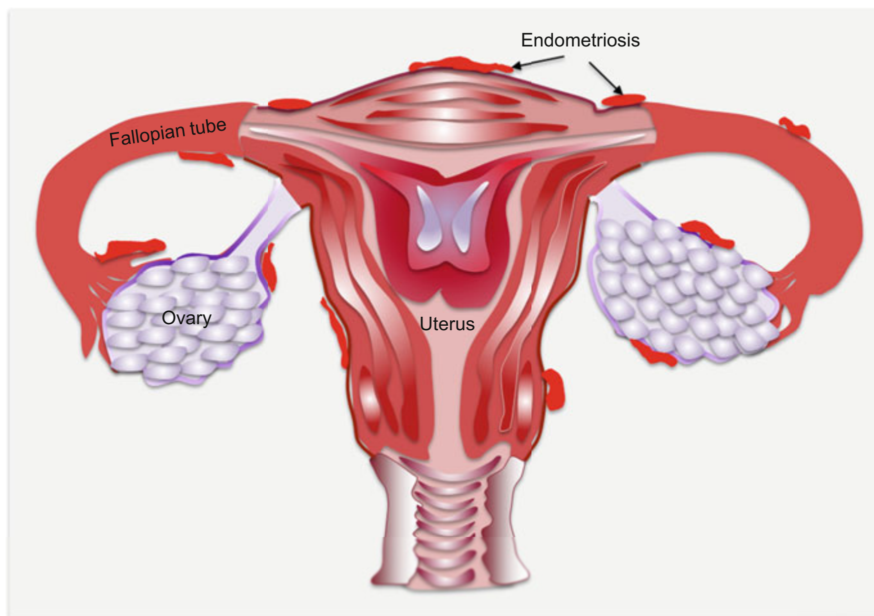

Fig. 14.1 Common anatomic locations of pelvic endometriosis (other common sites not shown are the “cul de sac,” bladder, and broad ligament)

endometriomas and filmy or dense adhesions. Using these criteria, there are four stages of endometriosis:

- Stage I or minimal disease is characterized by a few superficial implants.
- Stage II (mild) or disease is defined by more lesions that are slightly deeper implanted than lesions in stage I.
- Stage III or moderate disease has many lesions deeply implanted, in association with small endometriomas, and some filmy lesions.
- Finally, the severest stage IV lesions consist of many deeply implanted lesions in association with large endometriomas and many dense adhesions.

This classification helps guide clinical decision-making on patient management.

Several mechanistic theories are provided for the emergence of endometriosis. As a complex disease, endometriosis may be mediated by multiple mechanisms. For instance, the pathogenic process of pelvic endometriosis may differ from the rare findings of extra-pelvic endometrial tissues. Thus, the diverse proposed mechanisms include the classic retrograde menstruation model due to uterine hyperperistalsis or dysperistalsis, coelomic metaplasia, cellular induction, and possible circulatory spread via either lymphatic or blood vessels.

14.3.1 Molecular Alterations in Endometriosis

Endometriosis is a hormonal disease due to its specific addiction to estrogen. However, the cell of origin demonstrates some aspects of the hallmarks of the cancer cell. Thus, alterations in the epigenome, genome, and transcriptome that reflect on cellular processes such as altered hormonal signaling, immunologic responses, angiogenesis, and oxidative stress are all features of this enigmatic disease.

14.3.1.1 Hormonal Alterations in Endometriosis

Part of the ability of endometrial cells and tissues to survive in ectopic locations depends on hormonal control, analogous to the regulatory roles in the normal endometrium. Unlike the normal phases of the menstrual cycle whereby hormonal control is physiologically regulated, in endometriotic tissues, cyclical hormonal control is disrupted. The endometriotic cells are estrogen loving and progesterone resistant, with altered prostaglandin levels and signaling. The dependence of endometriosis on the estrogen axis is revealed by the deregulation of several mediators of estrogen signaling in endometriotic tissues. ERβ is upregulated in endometriotic tissues, and this is associated with cell cycle deregulation and aberrant proliferation of endometrial tissues. Additionally, polymorphisms in the ERβ, specifically +1730G:A, increase the risk of endometriosis and associated infertility. Estradiol levels are elevated in menstrual blood of women with

endometriosis. A target gene of estradiol, *CYR61*, is also upregulated in endometrial tissues from women with endometriosis. Altered PGE2 levels also impart the hormonal derangement in endometriosis. In normal endometrium, PGE2 and estradiol levels are low. Additionally, in normal endometrium, increased progesterone production in the luteal phase is associated with increased HSD17B2 levels involved in estradiol metabolism to estrone. On the contrary, these physiologic processes are deranged such that in ectopic endometrial tissues, PGE2 and estradiol levels are high, HSD17B2 levels are low, and this is associated with resistance to progesterone-mediated metabolism of estrogen. These hormonal alterations are explored for medical treatment of endometriosis using estrogen suppression.

14.3.1.2 Epigenetic Alterations in Endometriosis

The hormonal derangement and other pathophysiology of endometriosis are partly due to epigenetic alterations in specific genes. Similar to the cancer cell, the progenitor endometriotic cell has to undergo similar hardships to establish a successful niche. This phenotypic change is mediated in part by deregulated gene expression, possibly as a result of epigenetic gene modification.

The epigenome in endometriosis is modified at the DNA, histone, and miRNA levels. In 2005, Wu et al. first demonstrated promoter hypermethylation of the homeobox gene, *HOXA10*, in eutopic endometrial tissues from women with endometriosis [1]. *HOXA10* has functions in uterine physiology, being elevated in endometrial tissues at the mid-secretory phase of the menstrual cycle, coincident with the possible implantation of a fertilized ovum. Functionally, HOXA10 regulates progesterone receptor cofactors (e.g., KLP9) to mediate endometrial responsiveness to progesterone. Thus, decreased expression of *HOXA10* in endometrial tissues from women with endometriosis could partly account for the associated infertility (lack of implantation). In support of endometriosis being a disease of epigenetic alteration is the overexpression of DNA methyltransferases (*DNMT1, DNMT3A*, and *DNMT3B*), in endometriotic tissues, which could mediate promoter hypermethylation of multiple genes. Thus, hypermethylation of progesterone receptor isoform B (*PR-B*) in endometriotic tissues is associated with progesterone resistance; reduced E-cadherin expression due to *CDH1* promoter hypermethylation favors detachment and invasiveness. As a disease addicted to hormonal influence, altered expression of steroidogenic genes underlies the pathology of endometriosis. The expressions of steriodogenic genes are repressed in normal endometrial tissues partly due to promoter hypermethylation of steriodogenic factor-1 (*SF-1*), a transcription factor responsible for their expression. In contrast, endometriotic stromal cells have promoter hypomethylation of *SF-1* leading to overexpression of these steriodogenic genes involved in estrogen biosynthesis. Finally, the overexpression of *ERβ* in endometriotic tissues is due to unregulated promoter hypomethylation.

14.3.1.3 Immunologic Alterations in Endometriosis

Endometriosis may also be a disease of awry immunology at the molecular level. First, IL-6 and some other cytokines are elevated in endometriotic tissues. Second, epidemiologic evidence indicates the association of endometriosis with other autoimmune disorders. There are increased rates of fibromyalgia, autoimmune thyroiditis, hypothyroidism, multiple sclerosis, alopecia universalis, allergies, asthma, and chronic fatigue syndrome among women with endometriosis. Similarly, SNPs associated with rheumatoid arthritis (*CCL21* rs2812378 and *HLA-DRB1* rs660895) are associated with endometriosis.

Autoantibodies are also observed in tissues and circulation of women with endometriosis. Autoantibodies against laminin-1 are found in a vast majority (~90 %) of lesions from infertile-associated endometriosis. Consequently, elevated levels of anti-laminin-1 antibodies are in body fluids (serum and peritoneal fluid) of women with endometriosis and this could serve as a diagnostic biomarker. Autoantibodies against carbonic anhydrase are common in autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus. In infertile women with endometriosis, circulating anti-carbonic anhydrase antibodies are equally present. Other autoantibodies implicated in endometriosis are those against endometrial transferrin and α-2-Heremans Schmidt glycoprotein.

14.3.1.4 Mediators of Angiogenesis and Oxidative Stress in Endometriosis

A feature of endometriotic tissues that mimics some solid tumors is the high levels of vascularization. This is a necessary requirement for the endometriotic cell to sustain its existence as it proliferates and grows into a solid mass in its new environment. While the molecular mechanisms involved in new blood vessel formation in endometriotic lesions are poorly understood, hormonal signaling and immune response have been implicated. Estrogen signaling is involved in neovascularization, while inflammatory and other immune cells such as macrophages and dendritic cells contribute to the pro-angiogenic endometrial tissue environment. Additionally, angiogenic factors including VEGF, tissue factor, and its receptors are upregulated in endometriotic lesions.

Another feature of endometriosis is oxidative stress, as is common in solid tumors. Elevated free radicals in association with decreased antioxidant mechanisms play a role in endometriosis. Consistently, biomarkers of oxidative stress are elevated in sera from women with endometriosis compared to those without. Similarly, there are lower levels of the antioxidant, vitamin E, and its extracellular binding protein, afamin, in peritoneal fluid from women with endometriosis.

The Warburg phenomenon of aerobic glycolysis is also a feature of endometriosis. TGFβ1 signaling induces transcription of glycolytic gene (*HIF-1α, PDK1, LDHA,* and *SLC2A1*) expression accompanied by elevated lactate in peritoneal fluid

and endometriotic tissues, and these levels are significantly higher than in eutopic endometrium from women without endometriosis.

14.3.2 Malignant Potential of Endometriosis

Although endometriosis is quite prevalent, only ~1 % of cases will undergo malignant transformation, which mostly involves the ovaries. Women with endometriosis have elevated risk (OR, 1.46) for developing ovarian cancer, and the prevalence of endometriosis is 39 % and 21 % in clear cell and endometrioid ovarian cancer, respectively. Thus, ovarian clear cell and endometrioid cancers account for ~76 % of all endometriosis-mediated ovarian cancer.

The molecular mechanisms that control the transformation of benign endometrial tissues into malignant tumors are not fully elucidated. However, epigenetic aberrations have been implicated. Hypomethylation of LINE1 and *RASSF2* inactivation through promoter hypermethylation are early events in malignant progression of endometriotic lesions. Other relevant genes involved in malignant transformation of ovarian endometriosis include *RUNX3* inactivation and loss of MMR gene *hMLH1* via promoter hypermethylation. Additionally, LOH at 10q23.3 and *PTEN* mutations occur in up to 56 % and 20 % of endometriotic cysts, respectively. Moreover, mutations in *PTEN* (~20 %) and *CTNNB1* (16–54 %), as well as *TP53* overexpression and mutations (42–63 %) and MSI (12–18 %), occur in ovarian endometrioid adenocarcinomas.

While it is not recommended to refer women with endometriosis for oophorectomy (as practiced in women with *BRCA* mutations), women with endometriosis require close surveillance for early cancer detection. Women at elevated risk of progression are those who are diagnosed at an early age, and/or with long-standing histories of endometriosis, as well as women with associated endometriomas.

14.4 Endometriosis Biomarkers in Circulation

Noninvasive biomarkers are needed to curtail the morbidity associated with endometriosis. Towards achieving this has been the efforts at uncovering circulating endometriosis biomarkers. Of interest, changes in miRNA, immunologic and angiogenic factors, novel serum, and urine proteomics are all potential noninvasive biomarkers of endometriosis. The levels of ccfDNA are significantly higher in women with endometriosis than healthy controls ($p = 0.046$), suggesting the possible targeting of endometriosis-specific genetic alterations in circulation [2].

14.4.1 Circulating Endometriosis miRNA Biomarkers

MiRNA deregulation in the peripheral circulation as a diagnostic biomarker of endometriosis has been evaluated. Elevated in circulation of patients are miR-16, miR-122, miR191, miR-195, and miR-199a, while decreased are miR-9*, miR-17-5p, miR-20a, miR-22, miR-141*, miR-145*, and miR-542-3p [3, 4]. Wang et al. profiled and validated a select set of diagnostic serum miRNAs in women with endometriosis [3]. Levels of serum miR-199a and miR-122 were elevated, while miR-9*, miR-141*, miR-145*, and miR-542-3p were reduced in women with endometriosis. A panel composed of miR-199a, miR-122, miR-145*, and miR-542-3p achieved a diagnostic sensitivity, specificity, and AUROCC of 93.22 %, 96.00 %, and 0.994, respectively, for endometriosis. Additionally, the elevated levels of miR-199a and miR-122 could differentiate between women with mild and severe endometriosis, while miR-199a levels correlated with possible disease severity in terms of pelvic adhesion and lesion distribution. Focusing on let-7 family members, Cho et al. demonstrated that let-7b, let-7d, and let-7f levels were reduced in serum samples from women with endometriosis [5]. During the proliferative phase of the endometrial cycle, levels of Let-7 b, let-7c, let-7d, and let-7e are significantly reduced in circulation of these patients. A panel of let-7b, let-7d, and let-7f (at proliferative phase of the cycle) achieved an AUROCC of 0.929 in detection of endometriosis. The circulating levels of let-7b correlate with CA125, and as a single biomarker achieved an AUROCC of 0.692.

14.4.2 Circulating Endometriosis Protein Biomarkers

Proteomic approaches have enabled the use of serum spectral peaks to discriminate between women with and without endometriosis. Additionally, several circulating proteins, including glycoproteins, cytokines and other immune mediators, growth and angiogenic factors, adhesion molecules, and mediators of redox status have all been evaluated as potential diagnostic biomarkers of endometriosis. While there are currently no validated and hence commercially available circulating biomarkers for endometriosis, some of the findings show great promise.

14.4.2.1 Circulating Endometriosis Proteomic Spectral Peak Biomarkers

Proteomic profiling of samples to uncover diagnostic signatures of endometriosis has demonstrated potential. Jing et al. used SELDI-TOF MS to identify two serum proteins peaks that were associated with endometriosis [6]. As diagnostic biomarkers of endometriosis, these achieved a sensitivity of 86.67 % and specificity of 96.7 %. Importantly, these peaks declined to levels in control women following

surgery. Zheng and colleagues uncovered three peptide peaks that were used in a diagnostic model to detect endometriosis [7]. This peptide-peak model achieved a sensitivity of 91.4 % and specificity of 95 % when applied to women with ($n = 126$) and without ($n = 120$) endometriosis. In an independent validation set, this model performed at a similarly high sensitivity and specificity of 89.3 % and 90 %, respectively, in detecting endometriosis. Another study using SELDI-TOF MS uncovered five peptide peaks that achieved a sensitivity of 88% and specificity of 84 % in stratifying women with and without endometriosis [8]. Thirteen and twelve peptide peaks were found deregulated in women with endometriosis and adenomyosis, respectively, when compared to healthy controls. Five common peaks were decreased in both patient groups, and the two conditions could not be separated based on these serum peptide peak profiles [9].

14.4.2.2 Circulating Endometriosis Glycoprotein Biomarkers

Currently, the only circulating biomarker used by some centers to help detect endometriosis is CA125. Circulating CA125 is well studied as a diagnostic biomarker of endometriosis. It has demonstrated clinical utility in endometriosis management, but the sensitivity limits its general recommendation as a screening biomarker. A meta-analytical study revealed a sensitivity of 50 % at a specificity of 72 % for all stages of endometriosis, with a slight increased performance in women with late stage III/IV disease (sensitivity of 60 % and specificity of 80 %) [10]. Other members of the CA family, especially CA19-9 and CA15-3, are significantly elevated in circulation of women with endometriosis. In the analysis by Tuten et al., CA125 achieved a better AUROCC of 0.928 for the detection of endometriosis; however, levels of CA19-9 and copeptin were significantly much higher in stage III/IV than stage I/II disease patients [11]. Copeptin levels correlated with disease burden in terms of stage and size of endometriosis.

Aside from CA, many other glycoproteins are evaluated singly as potential diagnostic biomarkers. These include glycodelin (sICAM-1), follistatin, Zn-α2-glycoprotein, and several others. Serum levels of glycodelin failed to delineate adolescent women with endometriosis from those without [12]. However, in women with endometrioma, glycodelin was elevated in patients compared to controls and achieved a diagnostic sensitivity and specificity of 82.1 % and 78.4 %, respectively [13]. The levels of circulating follistatin, an inhibitor of activin, were elevated in women with endometriosis [14], but this data could not be reproduced [15]. In a pilot study using mass spectrometry, Zn- α2-glycoprotein was identified as differentially expressed between cases and controls. An ELISA assay was developed for this biomarker and confirmed the significant differential expression in women with and without endometriosis ($p = 0.019$). When applied to samples from 120 cases and controls ($n = 20$), this assay could detect endometriosis at a sensitivity of 69.4 % and specificity of 100 % [16]. A panel of biomarkers including glycodelin, annexin V, VEGF, and CA125 achieved a sensitivity of 74–94 % and specificity of 55–75 % in a training set [17].

14.4.2.3 Circulating Endometriosis Immunologic Biomarkers

As a disease of inflammation, circulating levels of immunologic mediators have been explored as biomarkers of endometriosis. Cytokines, chemokines, and cells of the immune system have all been explored as diagnostic biomarkers with mixed outcomes. With the exception of RANTES, IL-4, IL-8, MCP-1, TNF-α, YKL-40, and copeptin, many immunologic circulating biomarkers have demonstrated no discrimination between women with and without endometriosis. In adolescent women with endometriosis, IL-4 is significantly elevated in their sera [18]. Tuten et al. performed a pilot study of the circulating levels of the inflammatory mediator, copeptin, between cases and controls [11]. Levels were significantly increased in cases compared to controls. As a potential circulating biomarker of endometriosis, copeptin performed at a sensitivity and specificity of 65 % and 58.3 %, respectively, when using a cutoff value of 251.18 pg/ml. Another inflammatory biomarker identified by Tuten et al. is YKL-40, which is significantly elevated in women with and without endometriosis [19]. In a comprehensive review by May et al., significant increases in circulating levels of RANTES (concordant in 75 % of the studies), MCP-1 (concordant in 50 % of the studies), and IL-8 (concordant in 46.1 % of the studies) were uncovered in women with endometriosis, and may have potential as early detection biomarkers, especially if used in a panel [20]. A panel of three plasma biomarkers including IL-8, TNF-α, and CA125 impressively achieved a sensitivity and specificity of 89.7% and 71.1%, respectively, in differentiating between women with ($n = 201$) and without ($n = 93$) endometriosis [21].

Other immune mediators, specifically antibodies against multiple self-antigens, are found elevated in circulation of women with endometriosis compared to those without. Anti-endometrial antibodies in particular are frequently increased in patients than controls. Ovarian endometrioma is associated with elevated circulating anti-IMP-1 (insulin-like growth factor 2 mRNA-binding protein 1). Nabeta et al. uncovered high levels of serum anti-PDIK1L and anti-syntaxin 5 in patients with endometriosis [22, 23]. Additionally, circulating levels of antibodies against stomatin-like protein 2 (SLP2), tropomodulin 3 (TMOD3), and tropomyosin 3 (TPM3) are much higher in women with than without endometriosis. Other elevated serum levels of autoantibodies in women with endometriosis include cardiolipin, laminin-I, carbonic anhydrase, copper oxidized low-density lipoprotein, transferrin α2-HS glycoprotein, and lipid peroxide-modified rabbit serum albumin.

Levels of circulating immune cells have also been examined as potential biomarkers of endometriosis. A number of circulating immune cells (NK, T, and B cells, macrophages, and monocytes) have been studied with no defined utility as biomarkers. However, Olkowska-Truchanowicz et al. demonstrated decreased levels of $CD25^{high}$ foxhead box 3 positive ($FOXP^{+}$) subset of $CD4^{+}$ regulatory T cells in patients compared to controls [24].

Because inflammation and immunologic reactions are not specific to endometriosis, their role in delineating this disease from other inflammatory pathologies is

unclear. Indeed, a set of serum proteomic peaks failed to differentiate between women with endometriosis and those with adenomyosis.

14.4.2.4 Other Circulating Endometriosis Protein Biomarkers

Several cell adhesion molecules, angiogenic and growth factors, as well as markers of redox state are deregulated in women with endometriosis, and these have been assayed in circulation as potential biomarkers for disease detection. The levels of sICAM-I (glycodelin) increase early in women with endometriosis, while significantly elevated circulating levels of osteopontin, MMP-2, and MMP9 are observed in this disease.

Angiogenic and growth factors are elevated in circulation of women with endometriosis. Some biomarkers (e.g., VEGF, HGF) are, however, not reproducible or have conflicting reports. But other biomarkers such as sVEGFR-1 (Flt-I), FGF2, and angiogenin are elevated in patient samples. The hepatocyte growth factor receptor, cMET, is significantly more increased in sera from women with than without endometriosis [25]. Chen et al. demonstrated that the anti-inflammatory protein and inhibitor of angiogenesis, pigment epithelium-derived factor (PEDF), was significantly decreased in patients compared to controls [26].

Elevated redox-mediated molecules in circulation of women with endometriosis include HSP70b, lipid peroxidases, and vitamin E, while decreased circulating levels include superoxide dismutase and paraoxonase (PON-1).

14.4.3 Circulating Endometriosis Metabolomic Biomarkers

Several metabolites are deregulated in women with endometriosis and are evident in the peripheral circulation as well. In a pilot study of women with mild endometriosis compared with healthy control women, several metabolites were identified. Circulating levels of L-alanine, L-valine, L-leucine, L-threonine, L-lysine, lactate, 2-hydroxybutyrate, 3-hydroxybutyrate, glycerol phosphatidylcholine, and succinic acid were elevated, while glucose, L-arginine, and L-isoleucine levels were lower in patients compared to controls [27]. Vouk et al. compared serum metabolite profiles between women with and without endometriosis and uncovered significant elevated levels of 8 metabolites and 81 metabolite ratios in women with endometriosis [28]. Using an algorithm adjusted for age and BMI, the ratio of phosphatidylcholine C36:2 to ether-phospholipid C34:2, in addition to levels of hydroxy sphingomyelin achieved a sensitivity of 90.4 % and specificity of 84.3 % for distinguishing between women with and without endometriosis. Khanaki et al. found significantly decreased levels of stearic acid in patients with endometriosis [29].

14.4.4 Endometriosis Extracellular Vesicles

Apart from their biomarker potential, exosomal contents may play a role in the pathophysiology of endometriosis. Compared to simple ovarian cyst fluids, fluids from endometriomas had much increased ecto-nucleotidase activities, with those of ATPase being 5.5-fold and ADPase at 20-fold increase [30]. Importantly, ecto-nucleotidase-bearing exosomes were present in cyst fluid, suggesting their use in mediating the pathogenesis of endometriosis. Microvesicular cargo may also be involved in extracellular matrix (ECM) remodeling in endometriosis. Microvesicles, probably from human uterine epithelial cells (HESs), contained extracellular matrix metalloproteinase inducer (EMMPRIN) [31]. EMMPRIN induces human uterine fibroblast production of MMP1, 2, and 3, which are involved in ECM remodeling. The deregulated estradiol levels in endometriosis may induce the release of microvesicles by HESs. Conceivably, there are potentially several other microvesicular contents involved in the development of endometriosis.

14.5 Endometriosis Biomarkers in Other Body fluids

14.5.1 Endometriosis Biomarkers in Urine

Because of the reduced complexity of urinary proteome, a number of studies have explored urinary proteomics to identify endometriosis biomarkers. Moreover, the unique stability of the urinary proteome makes it attractive for biomarker exploration. Urinary proteome can be stable for close to two decades when stored at −70 °C and for several days at room temperature. MS-based urinary proteomics of samples from women with endometriosis compared to those without indicate differential protein levels between the two groups. Using MALDI-TOF MS, a 3280.9 Da peptide could differentiate between patients and controls at a sensitivity of 82 % and specificity of 88 % [32]. MALDI-TOF MS coupled with 2D-PAGE enabled the identification of five peptides highly elevated in urine from patients compared to controls [33]. Other biomarkers significantly elevated in urinary samples from patients include α-1 antitrypsin, prealbumin, vitamin D-binding protein, and enolase-1 [34, 35]. Urinary enolase-1 as a single diagnostic biomarker achieved a suboptimal sensitivity of 56 % and specificity of 72 %.

Using an ELISA, Cho et al. demonstrated increased urinary sFlt-1 in women with endometriosis [36]. MMP-2 and MMP9 levels were significantly elevated in urine from women with endometriosis, so was the ratio of MMP-9/neutrophil gelatinase-associated lipocalin [37]. While there are differential levels of CK19 between women with and without endometriosis, in a larger study, these were not proven to be of diagnostic utility as a urinary biomarker [33, 38].

14.5.2 Endometriosis Biomarkers in Peritoneal Fluid

Pathogenic factors in peritoneal fluids from women with endometriosis may contribute to the genesis of endometriosis. While minimally invasive, should an accurate peritoneal fluid biomarker be available for endometriosis, samples can be collected via transvaginal ultrasound-guided aspiration. These biomarkers have been explored for their clinical utility and biologic activity in women with endometriosis.

Because the molecular pathology of endometriosis involves altered immunity, several immunologic biomarkers have been evaluated in peritoneal fluids. Using cytokine array approach, numerous cytokines demonstrated differential expression in peritoneal fluids between patients and controls. For example, seventy-four elevated and four reduced cytokines demonstrated threefold change between patients and controls. Pathway analysis indicated many of these cytokines were involved in cell–cell interaction, adhesion, and protein synthesis. Of relevance activin A, SMAD7, and β-nerve growth factor were the most informative in regard to endometriosis pathogenesis [39]. Cytokines including IL-6 levels are significantly elevated in peritoneal fluids from patients, and this has been associated with pain. Additionally, peritoneal fluid IL-8 levels are elevated and correlate with CA125 levels and recurrence of endometriomas [40]. Peritoneal fluids NK cells from women with severe endometriosis express significantly high levels of TNFα and IFNγ than control women. Pathophysiologically, these may mediate angiogenesis, proliferation, and growth of endometriotic implants [41]. Peritoneal inflammation in endometriosis is partly mediated by IL-1. Consistently, IL1β levels are elevated, while levels of its inhibitors, soluble IL1 receptor accessory protein (sIL1RAcP) and soluble IL1 receptor type 2 (sIL1R2), are significantly reduced in peritoneal fluids from women with endometriosis [42]. While CD25+/FOXP3+ Treg levels were decreased in peripheral circulation of women with ovarian endometriomas, there were significantly elevated levels in peritoneal fluids of these patients. Thus, in women with ovarian endometriomas, Treg-mediated immune disruption may partly augment the development of pelvic autoimmunity [24]. The immunopathogenesis of endometriosis involves neutrophils and their peptides. Thus, human neutrophil peptides 1, 2, and 3 (HNP1-3) levels were significantly elevated in peritoneal fluids from patients, and these correlated with disease severity, and the elevated levels of neutrophils, T-cells, and IL-8 [43].

14.5.2.1 Endometriosis Diagnostic Biomarkers in Peritoneal Fluids

In adolescent girls with endometriosis, IL-6, TNFα, and glycodelin A levels in peritoneal fluids were significantly much higher than control girls without endometriosis. The odds for predicting endometriosis in this cohort at defined cutoffs of peritoneal fluid levels were 10.2 for IL-6 at 90.0 pg/mL, 14.6 for TNFα at 3.0 pg/mL, and 2.2 for glycodelin A at 60.0 ng/mL [12]. In peritoneal fluids from women

with moderate to severe endometriosis, IL-6 and IL-10 were significantly elevated. However, as a diagnostic, ROC analysis identified only IL-6 to be of value with AUROCC of 0.90 [44]. The levels of three chemokines, CXCL8/IL-8, CCL2/MCP-1, and CCL19/MIP-3β, were elevated in peritoneal fluids from women with endometriosis. The sensitivity of these three as a panel was 89.1 %, which significantly improved the likelihood of identifying women with endometriosis [45]. In women with endometriosis, the levels of IL-6, IL-8, and glycodelin A were elevated in peritoneal fluids. Diagnostic models based on biomarker ratios (biglycan to glycodelin A, ficolin-2 to glycodelin A, IL-8 to total proteins (in mg), and regulated on activated T-cell expressed and secreted to IL-6) in combination with age could detect women with endometriosis irrespective of menstrual phase. These biomarkers achieved ranges of diagnostic sensitivity, specificity, and AUROCC of 72.5–84.2 %, 78.4–91.2 %, and 0.85–0.90, respectively [46].

Other peritoneal fluid biomarkers include alterations in redox species, apoptotic markers, and MS peptide peaks. Redox stress is another hallmark of endometriosis. Compared to women with benign ovarian pathologies, women with endometriosis had significantly increased levels of oxidative stress markers such as 8-hydroxy-2-deoxyguanosine and 8-isoprostane in peritoneal fluid. These increased levels were associated with advanced stage disease [47]. While not cancerous, endometriotic cells must avoid apoptosis in order to implant and grow at their ectopic sites. Analysis of apoptosis markers in peritoneal fluid mononuclear and endometriotic cells revealed decreased levels of mFasL-bearing mononuclear cells and proteins with disease severity, while FasL transcript and protein levels were elevated. Thus, sFasL levels also increased with disease progression. These findings are indicative of decreased apoptotic ability of mFas-bearing mononuclear cells, which may additionally be targets of FasL-expressing endometriotic cells [48]. While not identified, studies of peritoneal fluid from women with endometriosis reveal differential MS protein peaks at m/z 4428, 6427, 6891, and 13,766 [49]. Moreover, 16 significantly differential peaks in peritoneal fluid samples achieved a sensitivity of 70.6 % and specificity of 80.8 % for endometriosis [50]. Other peritoneal fluid peptides for discriminating mild from severe endometriosis women have also uncovered [51].

14.6 Summary

- Endometriosis is a debilitating disease of women in their reproductive ages.
- It is a major cause of infertility and exerts substantial stress on global health economy.
- Genetic composition, nulliparity, pelvic inflammation, and obstruction to menstrual flow are possible risk factors of endometriosis.
- The pathophysiology of endometriosis involves a communication network among environmental, epigenetic, genetic, hormonal, redox, and immunologic factors.

- About 1 % of endometriosis progresses to malignancy that often involves the ovaries.
- MicroRNAs are potential noninvasive biomarkers of endometriosis.
- Proteomic technologies have also enabled the identification of circulating proteins, including glycoproteins as endometriosis biomarkers.
- Circulating inflammatory mediators are other potential endometriosis biomarkers.
- Urinary proteome offers another source of mining for discriminatory endometriosis biomarkers.
- Although peritoneal fluid acquisition is invasive, biomarkers in this medium may offer increased specificity for endometriosis.
- Given the global economic burden of endometriosis, noninvasive biomarker products are needed for early detection and to guide effective therapy.

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Chapter 15
Endometrial Cancer Biomarkers in Circulation

Key Topics

- Molecular pathology of endometrial cancer (EnCa).
- Circulating EnCa miRNA biomarkers.
- Serum CA125 and HE4 as EnCa biomarkers.
- Circulating adipocytokines as EnCa biomarkers.
- Circulating EnCa cells.

Key Points

- EnCa is a disease of postmenopausal women that is increasing in incidence. Mortality is, however, low because the majority present with low-grade tumors.
- The two types of EnCa have distinct and overlapping molecular pathology, thus requiring molecular subclassification. Data from The Cancer Genome Atlas uncovers four such subclasses with prognostic relevance.
- Circulating EnCa biomarkers include miRNAs, serum CA125, HE4, and YKL40, and adipocytokines. Emerging noninvasive biomarkers are those from serum proteomics and circulating EnCa cells.

15.1 Introduction

Endometrial cancer (EnCa) is the most commonly diagnosed genital cancer of women. Globally, 319,498 new cases were estimated with 76,160 deaths in 2012. The expected incidence and mortality for the US in 2016 are 60,050 and 10,470, respectively. About 75 % of all cases are detected in postmenopausal women, mostly in their 6th and 7th decades. As an age-associated disease, the incidence

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has been on the rise over the past few decades, probably mirroring increasing longevity. There are different geographic distributions of cases as well. The age-adjusted incidence rates are highest in North America, Europe, Australia, and New Zealand with the lowest in African and Asia.

A major risk factor for EnCa is hormonal stimulation of the endometrium, which may occur in conditions such as polycystic ovarian disease, estrogen-producing tumors, and unopposed estrogen therapy. Tamoxifen treatment of breast cancer is associated with increased risk, so is disturbed hormone metabolism associated with obesity. Hypertension and diabetes are also recognized risk factors. Finally, hereditary non-polyposis colorectal cancer confers hereditary risk for EnCa.

There are two pathologic subtypes of EnCas, designated types I and II. A majority (~80 %) of EnCas are histopathologic type I cases, which are associated with favorable outcomes. Although in the minority, type II EnCas account for ~50 % of all recurrences and the associated mortalities. The two types have distinct and overlapping molecular pathology. For example, *TP53* mutations are late events in type I, but early in type II disease. There is the need for actionable biomarkers for EnCa management, especially the more aggressive subtypes.

Noninvasive biomarkers will be useful in the clinical management of EnCa for a number of reasons. First, they will be optimal for early detection, especially of the more aggressive type II. Second, because EnCa is diagnosed mostly in older women, many patients may be frail and hence not suitable for serial invasive endometrial sampling for longitudinal monitoring. Finally, noninvasive biomarkers will help early detection of recurrences for expedited curative-intent therapies.

15.2 Screening Recommendations for EnCa

Screening either by transvaginal ultrasound or endometrial sampling of asymptomatic women is not recommended, because there is no evidence of reduced mortality and the harms may outweigh the benefits. False-positive ultrasound results, for instance, may cause unnecessary anxiety and trigger biopsy that may be associated with bleeding, infections, and discomfort.

15.3 Molecular Pathology of EnCa

Two main types of EnCa are recognized based on epidemiology, histopathology, molecular genetics, and clinical course. The majority of EnCas are carcinomas; however, a few variant forms such as carcinosarcomas and undifferentiated tumors are also recognized. The majority (70–80 %) of EnCas are categorized as type I or endometrioid endometrial cancer (EEC), while the remaining 10–20 % constitute type II or non-endometrioid EnCa (NEEC). Type I EnCas are low-grade tumors that develop as a consequence of estrogen exposure. The risk factors for these tumors

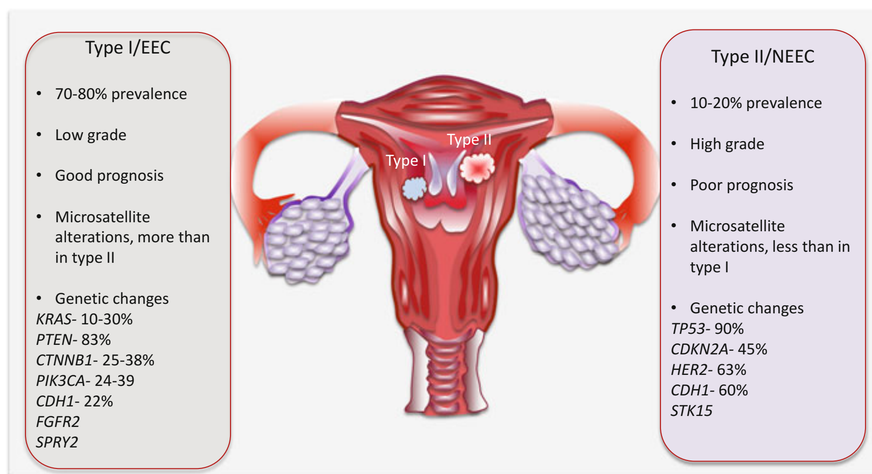

Fig. 15.1 Features and molecular pathology of types I and II EnCas

include nulliparity, anovulation, obesity, and exogenous estrogen exposure. Thus, these tumors express estrogen and progesterone receptors. Their progressive course may involve an initial intraepithelial atypical or complex hyperplasia. As low-grade (grade 1 and 2) tumors, they are associated with good prognosis. Type II EnCas are not estrogen responsive and histologically are high-grade tumors. They include grade 3 EEC as well as non-endometrioid serous, clear cell, squamous, transitional cell, mucinous, mesonephric, and undifferentiated tumors. These tumors are associated with adverse prognosis. A quick review of the features and molecular pathology of the two types is illustrated in Fig. 15.1.

15.3.1 Type I EnCa

Type I EECs are primarily characterized by alterations in microsatellite loci (MSI), *PTEN*, *KRAS*, *PIK3CA*, and *CTNNB1* among others. Notable alterations in these tumors include:

- Loss of *PTEN* occurs in the majority (~83 %) of EEC and in ~55 % of their precursor lesions, suggesting an early event in tumor progression. *PTEN* loss of function is primarily due to somatic mutations (37–61 %) and less so due to LOH at 10q23.3 (*PTEN* locus). *PTEN* LOH occurs in ~40 % of all EnCas. In EEC, *PTEN* mutations are increased up to 60–86 % on the background of MSI. Loss of *PTEN* functions causes endometrial cell growth, spreading, migration, and escape from apoptosis via deregulation of the PI3K and MAPK pathways.

- Mutations in *PIK3CA*, which encodes the catalytic subunit (p110α) of PI3K, cause deregulation of the PI3K pathway that is demonstrated in 24–39 % of EEC. The mutations occur more frequently at the helical (exon 9) and kinase (exon 20) domains and tend to coexist with *PTEN* inactivation. The kinase domain mutations are particularly associated with aggressive tumor behavior indicated by high-grade tumors with myometrial invasion and hence with adverse patient outcomes. Mutations in *PIK3CA* also occur in NEECs and as will be expected in mixed EECs and NEECs.
- *KRAS* mutations are more common in EECs (10–30 %) than NEECs (0–5 %) and are often associated with microsatellite unstable tumors. The RAS pathway negative effector, *RASSF1A*, is also frequently inactivated in EnCas via promoter hypermethylation.
- Gain-of-function mutations in *CTNNB1* are demonstrated in 14–44 % of EnCas but disproportionately more frequently in EEC (25–38 %). Mutations, primarily in exon 3, lead to nuclear accumulation of β-catenin and subsequent canonical WNT signaling. Tumors with *CTNNB1* mutations tend to lack MSI, *PTEN*, and *KRAS* mutations. The mutations are likely early events in disease progression because they are present in endometrial hyperplasia with squamous metaplasia (morules).
- An important signaling in EnCa is FGF pathway. The negative regulator of this pathway, *SPRY2*, is inactivated frequently via promoter hypermethylation in EnCas. Decreased immunoreactivity is demonstrated in ~20 % of EnCas. Additionally, somatic mutations of *FGFR2* are present in 6–12 % of EnCas, especially EECs. *FGFR2* mutations coexist with *PTEN* but are mutually exclusive of *KRAS* mutations.
- MSI is more associated with EECs than NEECs. In general, 25–30 % of all EnCas harbor MSI, but this is much higher (75 %) in EnCas arising on the background of HNPCC. MSI occurs commonly as replicative DNA repair errors secondary to loss of mismatch enzyme functions. Thus, the observed MSI in EnCa is a consequence of loss of *MLH1* due to promoter hypermethylation and, to a lesser extent, hereditary and somatic *MSH6* mutations. MSI is also demonstrable in precursor lesions, in association with *PTEN* loss, suggesting an early event as well. But these instabilities have been associated with tumor of high histologic grade. The DNA mismatch repair deficiency associated with MSI may underlie the increased mutations in other genes such as *PTEN*. Besides microsatellite sequence repeats located in noncoding regions, mononucleotide tandem repeats are also located in some genes such as *IGF2R, MSH3, MSH6, BAX, BCL10, APAF1,* and *ATR*, among several others that may be target of mutations as well.

15.3.2 Type II EnCa

Genetic alterations that are common, especially to serous NEECs, are *TP53* and *CDKN2A* inactivation, *HER2* amplifications, loss of *CDH1*, alterations in *STK15*

involved in the regulation of mitotic spindle, and chromosomal instability. Demonstrated alterations and their frequencies in these tumors include:

- The majority (up to 90 %) of serous NEECs harbor mutations in the tumor suppressor gene, *TP53*. Of interest, 80 % of the putative precursor lesions of serous carcinoma, endometrial intraepithelial lesions, also harbor these mutations.
- *CDKN2A* inactivation is very common in serous (45 %) and also some clear cell NEECs.
- *HER2* is amplified and overexpressed in 29 % and 43 %, respectively, of all NEECs, but in as many as 70 % and 45 %, respectively, of serous NEECs.
- *CDH1* loss or reduced expression of E-cadherin due to promoter hypermethylation or LOH at 16q22.1 characterizes NEEC. LOH is demonstrable in 66 % of NEECs compared to 22 % of EECs. The absence of E-cadherin is associated with poorly differentiated NEECs and poor prognosis.
- The mitotic spindle checkpoint involved in chromosomal segregation and centromere functions is important for normal cell division. Members involved in regulation of these spindle checkpoint activities including *BUB1, CCNB2,* and *STK15* are upregulated in NEECs. Indeed, frequent amplification of *STK15* that is involved in chromosome segregation is found in NEECs.
- Chromosomal instability, reflected by widespread chromosomal gains and losses and aneuploidy, is a molecular hallmark of NEEC.

15.3.3 The Need for Molecular Classification of EnCa

There is considerable overlap in the molecular features of the dichotomized types of EnCas. Additionally, some cases present with features of both EEC and NEECs and hence may demonstrate molecular features of both, although the predominant cell type may be overrepresented. Here are some reasons why molecular subtyping is important:

- While more frequent in one type, the molecular genetic alterations described above occur in both types of EnCas. For example, *PIK3R1* that encodes the inhibitory p85α subunit of PI3K is mutated in 43 % of EECs and 12 % of NEECs.
- Gene expression profiling of the PI3K pathway recognizes two different types of high-grade EnCas—those with PI3K pathway alterations and those with *TP53* mutations.
- While there are striking clinical and molecular genetic differences between clear cell and serous carcinomas, they are classified as NEECs due to their high-grade and aggressive phenotypes.
- While the molecular genetics of serous cancers from the endometrium and ovaries may be different, there are similarities between clear cell cancers from both organs. Mutations in *ARID1A* and loss of its encoded protein (BAF250a) are frequent in both clear cell NEECs and endometrioid ovarian carcinomas.

Indeed, gene expression profiles of clear cell carcinomas of the ovary and endometrium are similar, but those of serous endometrial and ovarian cancers are different.
- *ARID1A* mutations are also found in 29 % and 39 % of low-grade (grades 1 and 2) and high-grade (grade 3) EECs, respectively, as well as in 18 % and 26 % of serous and clear cell NEECs, respectively.
- Serous NEEC overexpress *CCND2A, IGF2, PTGS1*, and *FOLR,* while EECs overexpress a distinct set of genes including *MSX2, TFF3*, and *FOXA2*. Also, noted is the frequent downregulation of *SFRP1* and *SFRP4* by EnCas with MSI.
- It has been postulated that in mixed EEC and NEEC tumors, the NEEC component originates from EEC due to tumor progression. Thus, the presence of *PTEN, KRAS*, and *CTNNB1* mutations and MSI in some NEEC may be accounted for by this transition.
- To distinguish between high-grade 3 EEC and NEEC is very difficult, and it has even been shown that some grade 3 EECs harbor *TP53* mutations.
- Epithelial tumors that do not show squamous or glandular differentiation are referred to as undifferentiated carcinomas. They tend to grow in solid sheets and may have progressed from EEC because MSI is the main molecular feature of these tumors. However, some undifferentiated EnCas harbor *TP53* mutations suggestive of NEEC conversion.
- Uterine carcinosarcomas or sarcomatoid carcinomas (also known as malignant mixed mullerian tumors—MMMTs) are rare EnCas (<5 %). The molecular fingerprint of MMMTs suggests they are metaplastic tumors that emanate from endometrial cancers through EMT. It is believed that perpetual expression of EMT markers leads to loss of epithelial markers such as E-cadherin and acquisition of mesenchymal and sarcomatous histology. Indeed, EMT miRNA signature characterizes MMMTs, and two miRNAs (let-7b and Lin28B) frequently expressed by MMMTs and NEECs regulate *HMGA2* that is involved in EMT.

15.3.4 Molecular Classification of EnCa

Mining of The Cancer Genome Atlas data has enabled recognition of four subtypes of EnCas with prognostic significance. The four subtypes are characterized by:

- *POLE* mutations and their ultra-mutated phenotypes, which are associated with highly favorable clinical outcomes.
- Microsatellite instability (MSI).
- Copy-number low (CN-low).
- Copy-number high (CN-high), which consists mainly of high-grade serous carcinomas with poor prognosis.

While of enormous clinical relevance, routine translation or performance of these assays for molecular subtyping is not only laborious but also costly. To circumvent these issues, a group from the University of British Columbia has

developed surrogate markers that accurately mirror the molecular subgrouping using the TCGA data. These assays are easy to perform and hence can easily be clinically deployed. The assays are based on targeted mutational analysis, rather than whole genome sequencing, and immunohistochemistry (IHC) that can easily be performed cost-effectively. Features of this surrogate assay include:

- Replaces whole genome sequencing with targeted common *POLE* mutation analysis.
- Substitutes MSI assay with IHC targeting four MMR proteins (MLH1, MSH2, MSH6, and PMS2).
- CN-low and CN-high are differentiated by p53 IHC, whereby p53 wild type defines CN-low and abnormal expression identifies CN-high.

Thus, the surrogate subtypes are defined by:

- Targeted common *POLE* mutations.
- IHC for MLH1, MSH2, MSH6, and PMS2.
- Presence of wild-type p53, defined by p53 IHC score of 1+.
- Presence of abnormal p53 expression, defined by p53 IHC score of 0 or 2+.

15.4 Circulating EnCa Biomarkers

Circulating EnCa biomarkers include serum levels of CA125, HE4, YKL40, and adipocytokines. Other potential biomarkers are miRNA, serum proteomic spectra and novel proteins, as well as circulating EnCa cells.

15.4.1 Circulating EnCa DNA Biomarkers

A study of the role of circulating cell free DNA, *KRAS* mutations, and p53 antibody was conducted on 109 women with mostly type I EnCa ($n = 87$). Detected *KRAS* mutations and p53-Abs were stage dependent, increasing with advanced stage disease. However, ccfDNA and p53-Abs were mostly associated with high-grade tumors. These biomarkers may be of prognostic value [1].

15.4.2 Circulating EnCa MiRNA Biomarkers

A number of miRNAs show deregulated expression in EnCa tissue samples compared to normal endometrium. Oncomirs identified include let-7c, miR-7, miR-27, miR-34b, miR-103, miR-106a, miR-107, miR-181a, miR-185, miR-200b, miR-205, miR-210, miR-423, miR-429, and miR-449, while downregulated are the following tumor suppressormirs: let-7a, let-7e, miR-30c, miR-99b, miR-106b,

miR-129-2, miR-152, miR-193, miR-193b, miR-203, miR-204, and miR-221. The targets and roles of some of these miRNAs have been uncovered. For example, miR-27 promotes resistance to apoptosis by targeting *FOXO1*, while miR-200b promotes metastasis by decreasing the levels of TIMP2 to increase *MMP* expression. Enhanced tumor growth, progression, and metastasis are partly controlled by EnCa tumor suppressormirs. MiR-129-2, miR-152, miR-203, and miR-302 may prevent cancer cell survival, proliferation, and growth. SOX4, which induces EnCa cell growth, is a target of miR-129-2 and miR-203. MiR-302 targets *CCND1*, while miR-152 targets *mTOR, MET, E2F3*, and *DNMT1*. Metastasis-inhibitory miRNAs in EnCa include miR-30c, miR-106, and miR-204. MiR-106 prevents EMT by inhibiting *TWIST1*, miR-204 targets *FOXC1* to prevent metastasis, and miR-30c targets metastasis-associated gene 1 (*MTA1*). Thus, deranged miRNA expression promotes endometrial carcinogenesis.

Differential levels of circulating miRNAs are also demonstrated in women with EnCa. The levels of miR-155 are much higher in EnCa patients than controls [2]. There are stage-dependent significant levels, with higher circulating levels being found in patients with stage III and IV than stage I and II EnCa. Moreover, patients with lymph node metastasis harbored much higher levels of miR-155. Torres et al. analyzed *mTOR* target miRNAs (miR-99a, miR-100, and miR-199b) in tissue and plasma samples as possible cause of mTOR deregulation in eEnCa [3]. The three miRNAs were all downregulated in tissue samples in association with low circulating levels, and these were associated with increased *mTOR* expression. As diagnostic biomarkers, the three signature miRNAs in tissue as well as miR-99 and miR-199b in plasma were accurate at disease detection, while miR-100 was an independent prognostic biomarker of OS.

Profiling studies have also been applied to uncover differentially expressed miRNAs in EnCa tissue samples and in circulation. A follow-up study by Torres et al. of miRNA profiling of tissue and plasma samples uncovered altered expression of 17 miRNAs in eEnCa samples, with seven elevated and two reduced in plasma samples [4]. Tissue miRNAs alterations were of both diagnostic and prognostic value, with two miRNA signatures, miR-92a and miR-410 and miR-92a, miR-205, and miR-410, achieving diagnostic accuracies with AUROCC of 0.977 and 0.984, respectively. MiR-205 and miR-200a could predict relapse (AUROCC of 0.854). A signature composed of miR-1228, miR-200c, and miR-429 was an independent prognostic factor of OS (HR, 2.98), while PFS was associated with miR-1228 and miR-429 (HR, 2.453). Moreover, plasma miRNA levels were of diagnostic value with miR-9 and miR-1228 as well as miR-9, and miR-929 accurately detecting eEnCa with AUROCC of 0.909 and 0.913, respectively. Another global miRNA profiling of tissues and plasma with next-generation sequencing identified 11 miRNAs associated with eEnCa [5]. Three miRNAs (miR-499, miR-135b, and miR-205) were upregulated while five (miR-10b, miR-195, miR-30a-5p, miR-30a-3p, and miR-21) were downregulated in tumor tissues compared to normal controls. The diagnostic accuracy of two tissue miRNA signatures, miR-135b and miR-195 and miR-135b and miR-30a-3p, yielded AUROCC of 0.9835 and 0.9898, respectively. Plasma miR-135b and miR-205 levels were elevated, while miR-30a-3p and miR-21 were decreased. Following hysterectomy,

significant decreases in plasma miR-135b, miR-205, and miR-30a-3p were observed. As noninvasive diagnostic biomarkers of eEnCa, elevated levels of two single miRNAs, miR-135b and miR-205, achieved AUROCC of 0.9722 and 1.00, respectively. The screening of 375 miRNAs in plasma led to identification of differential circulating levels of miR-15b, miR-27a, and miR-223 in women with eEnCa [6]. As stand-alone biomarkers, the AUROCC for miR-15b, miR-27a, and miR-223 were 0.768, 0.813, and 0.768, respectively, for detection of eEnCa. In combination with serum CA125 levels, the performance of miR-27a was enhanced to AUROCC of 0.894. Thus, differential circulating levels of miRNAs hold clinical promise in EnCa worthy of further exploration.

15.4.3 Circulating EnCa Protein Biomarkers

As the most common cancer of the female genital tract, and yet with no screening recommendations for the broader population at risk (mostly postmenopausal women), noninvasive biomarkers for the early detection and management of EnCa are needed. Yet, there are currently no validated biomarkers for screening or accurate management of EnCa. A number of serum proteins are significantly elevated in EnCa patients compared to healthy control women. These include CA125, CA19-9 CA15-3, CA72-4, HE4, YKL-40, IL-6, MIP-1α, MIP-1β, TNFRI, IL-2R, IGFBP-I, TSH, Prolactin, GH, ACTH, TGFα, MMP-7, MICA, CEA, SCCA, SAA, and IAP, among several others. Significantly decreased circulating levels of Eotaxin, VEGF, ErbB2, EGFR, AFP, mesothelin, FSH, LH, CD40L, sVCAM-1, sICAM-1, tPAI-1, MPO, adiponectin, MMP-2, MMP-3, MMP-8, MMP-9, ULBP-1, ULBP-3, TTR, and sFasL in patients with EnCa compared to healthy individuals have also been uncovered [7]. Attempts have been made to evaluate the clinical utility of some of these serum biomarkers singly or in combinations for EnCa detection and management. Additionally, serum proteomic approaches have been employed for the discovery of discriminatory spectral peaks and novel proteins for EnCa.

15.4.3.1 Serum CA125 as EnCa Biomarker

CA125 is elevated in circulation of EnCa patients, and this has been explored primarily for the postdiagnostic patient management, because of the low sensitivity that precludes its use as a screening biomarker. Moreover, the same population at risk for EnCa is equally likely to develop ovarian cancer, whereby the diagnostic performance of CA125 is more accurate.

Serum CA125 is elevated (>35 U/ml) in 10–40 % of women with EnCa compared to healthy control women. Putatively, the low detection rate of CA125 in EnCa could be due to lack of release from cells as demonstrated in a small cohort of patients. In 28 EnCa patients, elevated serum CA125 was demonstrated in

21.4 % of cases in contrast to 89.3 % of matched tissue samples by IHC [8]. However, preoperative elevation of serum CA125 is associated with various clinicopathologic features of EnCa, including advanced FIGO stage, histologic grade, depth of myometrial invasion, lymph node metastasis, and peritoneal spread. Not surprisingly, therefore, a rising CA125 mirrors disease progression. With a cutoff value of 35 U/ml, elevated levels were demonstrable in 15.2 % of stage I, 33.3 % of stage II, 61.5 % of stage III, and 100 % of stage IV patients. Additionally, the likelihood of extra-uterine metastasis in patients with CA125 levels $\leq$20 U/ml was only 3 %. At this set cutoff (35 U/ml), the sensitivity and specificity for predicting advanced stage disease with adnexal involvement are 75 % and 69.5 %, respectively.

Elevated serum CA125 levels are potentially useful for recurrence and prognostic predictions. With the commonly used cutoff of 35 U/ml, levels above this are observed in 50 % of women with relapse, compared to 5.1 % of disease-free patients. In combination with CA19-9, recurrence prediction by CA125 achieved a sensitivity of 83.3 %, with 12.8 % false-positive rate [9]. As a prognostic indicator, elevated pretreatment levels are associated with poor outcomes. A highly elevated level >65 U/ml is significantly associated with shorter OS. Indeed, even levels >35 U/ml have significant correlation with cancer death and are independent prognostic predictors of adverse outcome in women with EnCa. A study involving analysis of ROC enabled the determination of CA125 cutoff at 25 U/ml as the best predictor of lymph node metastasis with a sensitivity and specificity of 78 % each for all patients and 71.6 % and 83.3 % for stage I patients [10]. For predicting the presence of adnexal involvement, CA125 > 30 U/ml yielded a sensitivity of 81 % at a specificity of 78.4 %. Increased CA125 was thus an independent predictor of poor prognosis.

Circulating levels of CA125 have been explored for other clinical uses such as potential for surgical planning. Sadowski et al. used a combination of serum CA125 and MRI to determine the likelihood of patients requiring lymphadenectomy at surgery [11]. These two biomarkers achieved a sensitivity, specificity, PPV, and NPV of 94 %, 91 %, 84 %, and 97 %, respectively. With only histologic grade as a predictor, positive lymph node rate was 4.0 %, but was increased to 11.1 % when positive MRI and elevated CA125 levels were considered.

Apart from being weary of the release of CA125 into the circulation by a number of other tumors, abdominal radiation can stimulate mesothelial cell release of CA125 as well. Caution is therefore needed in interpreting CA125 results in women who receive abdominal radiation prior to sample acquisition. Several other carbohydrate antigen biomarkers including CA15.3 and CA19.9 have been explored for EnCa with equally unimpressive diagnostic performances.

15.4.3.2 Serum HE4 as EnCa Biomarker

Human epididymis protein 4 (HE4) is elevated in women with early-stage EnCa and is diagnostically more sensitive than CA125. It has a high specificity (95 %) for

EnCa and is elevated in ~46 % (compared to 11–40 % for CA125) of cases. Similar to CA125, the elevated serum HE4 levels have potential utility in disease prognosis, stage prediction, and recurrence monitoring. Circulating levels may also have diagnostic potential in cancers with endometrioid histology. Minar et al. demonstrated significant differences in serum HE4 between women with eEnCa and those with benign endometrial pathology and healthy controls [12]. At a diagnostic cutoff value of 48.5 pmol/l, the sensitivity, specificity, and NPV were 87.8 %, 56.6 %, and 81.1 %, respectively.

The diagnostic utility of HE4 has been well studied and meta-analyses have been conducted. One analysis involved six studies including 791 cases and 760 controls [13]. The available studies had high level of heterogeneity. However, the performance of HE4 as a diagnostic biomarker of EnCa was modest. The pooled sensitivity, specificity, SAUROCC, and DOR were 59 %, 92 %, 0.833 and 20.82 respectively. The low sensitivity makes this unattractive as a screening biomarker. Two recent meta-analyses with more studies comparing HE4 to CA125 confirm the specificity of serum HE4 for EnCa. Despite study limitations (e.g., heterogeneity), Hu et al. concluded that HE4 may be superior to CA125 as a screening biomarker (SAUROCC for HE4 and CA125 were 0.7778 and 0.5474 respectively) [14]. Chen et al. supported this conclusion, and noted the enhanced performance when HE4 and CA125 were used as a panel biomarker (DOR and SAUROCC for the panel were 21.86 and 0.83 respectively vs. 17.01 and 0.77 respectively for HE4 alone) [15]. Thus, HE4 assay could be used as an ancillary to other diagnostic modalities in high-risk women.

HE4 has also been used for EnCa stage prediction. At a preoperative cutoff of 70 pmol/l, elevated HE4 levels correlated with histologic grade and stage of disease, with the highest circulating levels in patients with undifferentiated tumors. The increased levels also mirrored FIGO stage with detection frequencies of 42 %, 77 %, 90 %, 93 %, and 100 % in women with stages IA, IB, II, III, and IV, respectively [16]. Using ROC analysis, stage-specific cutoffs were determined. Stage-specific cutoffs and performances obtained were 61.3 pmol/l for stage IA (sensitivity and specificity of 82.3 % and 96 %, respectively), 89.2 pmol/l for stage IB (sensitivity and specificity of 83.3 % and 96 %, respectively), 104.3 pmol/l for stage II (sensitivity and specificity of 80.9 % and 98.6 %, respectively), 152.6 pmol/l for stage III (sensitivity and specificity of 92.5 % and 98.6 %, respectively), and 203.8 pmol/l for stage IV (sensitivity and specificity of 81.8 % and 99.3 %, respectively). In another study, HE4 levels were determined in women with eEnCa prior to surgery, and the levels correlated with disease stage, grade, and depth of myometrial invasion and was useful in predicting patients at high risk for having aggressive disease [17]. At a cutoff of 76.5 pmol/l, the sensitivity, specificity, and AUROCC for stage or risk for aggressive disease prediction was 72.4 %, 75.4 %, and 0.77, respectively. This was useful in preoperative staging of patients.

The potential of serum HE4 in predicting disease recurrence has been explored. HE4 levels decreased following treatment in responsive patients but was elevated >70 pmo/l in 81 % of cases with recurrences. When considering eEnCa, the AUROCC was 0.87 for recurrence prediction, with a sensitivity of 84 % and

specificity of 74 %. These parameters were much superior to elevated CA125 (>35 U/ml) that detected only 46 % of recurrences with AUROCC of 0.67 [18]. HE4 levels at diagnosis and disease recurrence were significantly different between patients with and without recurrence. This study could more accurately predict recurrence risk using high (201.3 pmol/l) HE4 levels at primary diagnosis, with sensitivity, specificity, PPV, and NPV of 80 %, 91 %, 90.3 %, and 90.8 %, respectively [19]. Expectedly, the performance was particularly better in patients with eEnCa.

HE4 may be a better predictor of prognosis in women with eEnCa than CA125. In women with stages III and IV cancer, HE4 was superior to CA125 in determining myometrial invasion (AUROCC was 0.77 vs. 0.65). In multivariate Cox analysis, HE4 was an independent predictor of recurrence-free survival (HR; 2.4) [20]. Serum levels were much higher in advanced than early-stage disease, and the elevated levels correlated with prognostic variables such as lymphovascular space, lower uterine segment, and endometrial stromal involvement, as well as deep myometrial invasion [21].

HE4 levels have also been useful in presurgical treatment planning as to the need or not for lymphadenectomy. An ROC analysis of preoperative levels in patients with early-stage disease placed the optimal cutoff at 78 pmol/l for accurate prediction of patients requiring lymphadenectomy, with sensitivity, specificity, PPV, NPV, and AUROCC of 86.6 %, 67.2 %, 51.2 %, 88.4 %, and 0.814, respectively. The high NPV is important for such assays that trigger invasive surgery when positive. The addition of CA125 (>26 U/ml) failed to improve the performance of HE4 [22].

15.4.3.3 Serum YKL40 as EnCa Biomarker

Circulating levels of the chitinase-3-like protein 1 (YKL40) have shown clinical promise in regard to disease detection and prognostication. In one series, YKL40 was elevated (>61 ng/ml) in 76 % of EnCas compared to 62 % for elevated CA125 [23]. The diagnostic utility of YKL40 was examined in a meta-analysis that evaluated seven studies involving 234 cases and 300 controls [24]. A modest diagnostic performance was uncovered with a pooled sensitivity, specificity, PLR, NLR, and DOR of 74 %, 87 %, 5.74, 30 %, and 19.14, respectively, and a SAUROCC of 0.80.

Peng et al. demonstrated the prognostic role of YKL40 in EnCa using tissue samples [25]. Positive immunoreactivity was associated with poor PFS and OS. In another cohort, preoperative levels were significantly higher in cancer patients than in women with uterine myomas, and the elevated levels declined following surgery. However, elevated YKL40 levels were associated with shorter PFS and OS. [26]. Preoperative median levels were as high as 137 ng/ml in women with EnCa compared with 28 ng/ml in controls [23]. Levels >80 ng/ml were associated with poor outcomes in univariate analysis. The 5-year survival rate was significantly better for patients with YKL40 levels <80 ng/ml (PFS and OS rates were

80 % and 79 %, respectively) than those with higher levels (PFS and OS rates were 43 % and 48 %, respectively).

It may be that apart from cancer, YKL40 is released into the circulation from other sources as well. Fan et al. found the staining rate to be much higher in patients (34.1 %) than control women with uterine myomas (11.1 %) [27]. However, in matched serum samples, the detection frequency among the patients was higher (63.4 %) than the corresponding tissues.

15.4.3.4 Serum EnCa Proteomic Biomarkers

Proteomics approaches have also been explored for discovery of circulating EnCa biomarkers. Yurkovetsky et al. uncovered a set of differential serum proteins between cases and controls, from which prolactin was identified as the strongest single discriminatory biomarker for EnCa, with a sensitivity of 98.3 % and specificity of 98 % [7]. Stage-dependent levels were different in regard to CA125, CA15-3, and CEA, which were higher in stage III than stage I disease. A panel consisting of prolactin, GH, Eotaxin, E-selectin, and TSH could accurately differentiate EnCa from ovarian and breast cancer women.

The use of MALDI-TOF MS analysis of serum samples enabled the identification of albumin-bound peptides for discriminating between EnCa and control women. Three peaks at *m/z* 4769, 6254, and 11792 could significantly detect EnCa with AUROCC of >0.8 ($p < 0.00001$). With a defined cutoff, the three peaks achieved a modest sensitivity of 65.2 % and specificity of 60.3 % for stage I disease, but they also detected 44.6 % of uterine myomas, thus negating their specificity for EnCa [28]. Serum samples from healthy controls and patients with either endometrial hyperplasia or cancer, depleted of high abundant proteins, were labeled with iTRAQ reagent and subjected to 2D LC-MS/MS. Seventy four proteins were identified of which 12 had a minimum of 1.6-fold change between patient and control samples. Of the 12 discriminatory proteins, 7 (ovosomucoid, haptoglobin, SERPINC1, α-1-antichymotrypsin, apolipoprotein A-IV, inter-α-trypsin inhibitor heavy chain H4, and histidine-rich glycoprotein) were novel biomarkers of endometrial hyperplasia [29]. Farias-Eisner et al. examined three OvCa biomarkers (apolipoprotein A1, transferrin, and prealbumin) in women with endometriod and papillary serous adenocarcinoma of the endometrium [30]. As a diagnostic panel, the three biomarkers accurately distinguished normal from early-stage disease samples at a sensitivity of 71 % and specificity of 88 % and from late-stage samples at similar performance of sensitivity and specificity of 82 % and 86 %, respectively.

15.4.3.5 Circulating Adipocytokine Levels as EnCa Risk Biomarkers

The associations of serum adipokine levels and the risk for developing EnCa have been explored. A prospective case–control study nested within the European

Prospective Investigation into Cancer and Nutrition found an inverse relationship between adiponectin levels and EnCa risk (OR 0.56), and this relationship is even stronger in obese, perimenopausal, and postmenopausal women [31]. These associations remained significant after adjustment for obese-related physiologic risk factors. On the other hand, in a similar prospective nested case–control study, there was no difference in adiponectin levels between cases and controls, and even levels above 15 μg/ml did not confer any risk for EnCa [32]. But Luhn et al. observed an inverse correlation between adiponectin levels and the risk of EnCa (OR, 0.48), while leptin levels were positively correlated (OR, 2.77) [33]. When considering postmenopausal women not on hormonal therapy, these associations were stronger and remained significant after adjusting for estradiol and BMI (OR 0.25 for adiponectin and 4.77 for leptin).

The many inconsistent findings were subjected to meta-analysis to determine the utility of adipocytokines in EnCa risk [34]. Of 14 manuscripts involving 1963 cases and 3503 controls, the SOR for EnCa risk was 0.47, 2.19, and 0.45 for adiponectin, leptin, and adiponectin/leptin ratio. This analysis also revealed an 18 % risk reduction for every 5 g/ml rise in circulating adiponectin levels (OR 0.82). No evidence of publication bias was detected. Thus, increased circulating adiponectin, adiponectin/leptin ratio, and/or decreased leptin levels confer reduced risk for development of EnCa.

15.4.4 Circulating EnCa Cells

Bone marrow aspirates were subjected to DTC detection using pancytokeratin antibody A45B/B3 [35]. Of 78 EnCa patients, the detection rate was 17 %, and this correlated with FIGO stage, lymph node involvement, and recurrence status. In a follow-up study, the detection rate was similar to the previous, at 16 % of the 141 patient samples analyzed. However, there was no correlation with clinicopathologic factors or clinical outcome [36]. In another study, DTCs were detected at a rate of 21 % (64/311) of EnCa patients [37]. Consistent with Banys et al., in multivariate analysis, DTC positivity did not significantly correlate with any clinicopathologic status. It was concluded that DTCs from EnCa have limited potential for metastatic regrowth.

The potential clinical roles of circulating EnCa cells (CEnCaCs) have been addressed. Peripheral blood samples from preoperative newly diagnosed EnCa patients with no evidence of disease following treatment and those with disease recurrence were subjected to *KRT20* PCR for CEnCaC detection [38]. CEnCaCs were positive in 35 % of the newly diagnosed, 51 % of those treated with no evidence of disease, and in all patients with recurrent disease. Blood samples from grade 3, stage Ib–IV patients and those with recurrent disease were subjected to CEnCaC isolation and detection using CELLection^{TM} Epithelial Enrich kit (Invitrogen, Dynal) and RTqPCR [39]. In contrast to DTCs, CEnCaCs demonstrated remarkable plasticity in regard to their stem cell phenotype, evidenced by

the expression of stemness markers, *ALDH* and *CD44*, as well as EMT markers, *ETV5, NOTCH1, SNAI1, TGFβ1, ZEB1*, and *ZEB2*. Potentially, these cells can establish metastatic deposits. In grade 3 EnCa patients undergoing surgery, preoperative blood samples were subjected to CEnCaC detection by immunomagnetic and immunofluorescence assays [40]. While the detection rate was dismal at 7 %, there was significant association with myometrial invasion and lymph node involvement. Notably only patients with endometrioid histology were positive for CEnCaCs by this assay, suggesting the need to identify other methods or markers, especially for the detection of type II EnCa circulating cells.

15.5 Summary

- Similar to ovarian cancer, EnCa is primarily a disease of postmenopausal women.
- A major risk factor of EnCa is hormonal stimulation of the endometrium.
- Although a continuous disease, there are two extreme histopathologic types of EnCa: type I or endometrioid EnCa and type II or non-endometrioid EnCa.
- Many (~80 %) EnCas are type I.
- The two types have distinct and overlapping molecular pathology.
- Examination of The Cancer Genome Atlas data uncovers four subtypes of EnCa with prognostic implications.
- MiRNAs play a role in the molecular pathology of EnCa and are emerging as circulating biomarkers.
- Serum CA125, HE4, and YKL40 are useful biomarkers for disease prognostication and staging.
- Serum proteomics are employed for discovery of early detection biomarkers.
- High circulating adiponectin and low leptin levels are associated with reduced EnCa risk.
- Circulating EnCa cells may hold prognostic potential, but this awaits technology development.

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Chapter 16
Cervical Cancer Biomarkers in Circulation

Key Topics

- Molecular pathology of cervical cancer (CvCa).
- Circulating CvCa and high-risk HPV DNA as CvCa biomarkers.
- Circulating CvCa miRNA biomarkers.
- Serum proteins as CvCa biomarkers.
- Circulating CvCa proteomic biomarkers.
- Circulating CvCa cells.

Key Points

- Globally, CvCa is the fourth most commonly diagnosed cancer in women, but in general is associated with good prognosis. Unfortunately, though, the vast majority is diagnosed in the resource-poor parts of the world and is associated with most of the fatalities.
- The major etiologic factor of CvCa is high-risk HPV infection of cervical epithelia cells. The pathogenesis is thus deregulation of signaling pathways by E6 and E7 viral oncoproteins.
- Established noninvasive biomarkers of CvCa include CA125, SCCA, and CYFRA 21-1. Circulating miRNAs are potential CvCa biomarkers; however, the role of serum proteomics and circulating CvCa cells awaits further discoveries and evaluation.

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16.1 Introduction

Cervical cancer (CvCa) is one of the three most commonly diagnosed tumors of the female reproductive tract. It is the fourth most common cancer in women with global estimates of 578,000 new cases and 260,000 deaths in 2012. Unlike other cancers, only 12,990 new cases and 4,120 deaths are estimated for the US in 2016. Due in part to active screening programs that enable early CvCa detection, the 5-year prevalence is as high as 1.55 million. Unfortunately, though, the global burden of CvCa is high in the less developed world, where ~ 85 % of all cases are diagnosed. The lack of early detection also partly account for the high mortalities associated with CvCa in these countries. Thus, 87 % of all CvCa deaths occur in the less developed world. Age-standardized rates of >30 per 100,000 are found in parts of Sub-Saharan Africa and Melanesia, with the lowest in Australia and New Zealand.

Although not sufficient as a causative factor of CvCa, high-risk HPV infection of the uterine cervix is an established risk agent of cervical epithelial cellular transformation. Heightened screening programs by Papanicolau (Pap) smear cytology beginning at age 21, coupled with HPV co-testing at 30, have translated into lowered incidence and mortality rates. Unfortunately, the achievements made by these screening efforts are realized only in the developed world where the annual incidence rates of CvCa are as low as 83,000 compared to the alarming rates of 445,000 in the less developed world. Expectedly, the ineffective screening in the developing world is associated with diagnosis of advanced stage disease, culminating in the high annual mortality of 230,000 cases, compared to the 35,000 deaths in the developed world.

The poor performance of the CvCa screening program in the developing world is due to myriads of factors including cultural, inaccessible, and inadequate healthcare services. Thus, accurate biomarkers in noninvasive clinical samples such as body fluids that can be deployed at the point-of-care through lab-on-a-chip technology should help curtail the disease burden in women in the resource-poor countries and communities.

16.2 Screening Recommendations for CvCa

Cancer of the uterine cervix is one of the few cancers where intense screening programs are in place. It is recommended to begin at age 21 for all women and continue till age 65. Pap smear cytology is the recommended screening method, and this should be performed at three yearly intervals beginning at age 21. Because cervical HPV infection is an etiologic factor, it is also recommended to include HPV testing beginning at age 30, and this Pap smear and HPV co-testing should be performed every 5 years. For women with normal screening results, it is not recommended to continue screening past age 65. However, for those with histories of CIN2 or higher in the last 20 years, continued surveillance is recommended.

16.3 Molecular Pathology of CvCa

Acquired knowledge on the molecular pathology of CvCa has led to the identification of viral and cellular biomarkers currently in clinical practice or being evaluated for translation. This session examines the molecular events in CvCa development and progression.

16.3.1 Types of CvCa

Any cellular composition of the uterine cervix can undergo transformation, hence the elaborate WHO classification of CvCas. However, the commonest cancers are cervical carcinomas, which either originate from precursor lesions in the squamous (squamous cell carcinoma—SCC) or glandular (adenocarcinomas) epithelial cells. The precursor lesions of SCC of the cervix are cervical intraepithelial neoplasia (CIN), types 1–3, and those of adenocarcinomas are adenocarcinoma-in-situ (ACIS). The majority of CvCas are squamous cell carcinomas (85 %), followed by adenocarcinomas (10 %) and the remaining 5 % constitute rare cancer types such as melanocytic and mesenchymal tumors.

16.3.2 Etiologic Agents of CvCa

HPV infection of the uterine cervix is the established etiologic agent of CvCa based on epidemiologic, clinical, pathologic, and molecular genetic evidences. Indeed, almost all SCCs (99.7 %) and adenocarcinomas (94–100 %) harbor HPV genomes. The risk of developing CvCa first depends on the type of HPV infection. Established data dichotomizes all HPVs (~120) into low-risk (LR-) and high-risk (HR-) HPVs. Low-risk or non-oncogenic HPVs (e.g., HPV6 and HPV11) tend to predominate in CIN1 lesions, whereas the oncogenic HR-HPVs are encountered more often in CIN3 lesions and CvCas, offering further support for their oncogenic functions. The majority (~53 %) of CvCas are caused by HR-HPV16, with the remainder being attributed to other HR-HPVs including HPV18 (15 %), HPV45 (9 %), HPV31 (6 %), and HPV33 (3 %). HPV infections are ubiquitous, with the cumulative lifetime risk for cervical infection of a woman being ~80 %. But the immune mechanisms of many infected women efficiently ward off the infections without any sequelae. However, ~20 % of HR-HPV-infected cases proceed to develop CIN1. Similarly, many of these lesions will not progress further. It is the few women who fail to fight off the infections and, hence, live with persistent HR-HPV infections that will progress to CIN3 and eventually develop CvCa.

16.3.2.1 HR-HPV and CvCa

Having succeeded in overcoming the host immune system, the molecular pathology of disease progression is mediated primarily by viral oncoproteins E6 and E7. Following entrance into the cervical squamous epithelium (due to failed barrier functions), HR-HPV infects the basal stem cells, and viral genomes or episomes can be visualized in the cytoplasm. As the stem cells differentiate and move towards the epithelial surface, viral encoded early genes, including E1, E2, E4, E5, E6, and E7) mediate viral genome transcription and replication to increase viral copy number. When close to the outermost layer, late viral genes, L1 and L2, are encoded to provide viral capsid proteins needed for the packaging and release of viruses in exfoliated epithelial cells. This cyclical process or productive infection is harmless to the infected host. However, in persistent infections, and under certain circumstances, loss of genetic control of viral genome transcription can lead to overproduction of early viral oncoproteins including E6 and E7, which can be pathogenic. In a nutshell, E6 and E7 mediate cellular escape from apoptosis and uncontrolled proliferation, immortalization, and eventual genomic instability with development of aneuploidy. These deregulated control mechanisms drive the development of dysplastic lesions and CvCa.

The success or failure to form dysplastic lesions that will progress to CvCa is the consequence of the interplay of three main factors. First, upon viral infection, the host immune system gets activated and attempts to mount an efficient immunity against the infection. In many cases, this is successful and the infection is cleared. Second, the infected viruses adopt several mechanisms to escape the immune system, rendering it inefficient in viral clearance. These mechanisms include downregulation of cytokine production and inhibition of type I interferon pathways to reduce interferon-mediated gene expression that can establish the antiviral state. Finally, host factors play a role in efficient viral clearance. For example, the HLA allele DQB1*03 is associated with increased risk, while allele DRB1*13 confers reduced risk for developing CIN lesions. Thus, the dynamic interplay between HR-HPV infection, immune mechanisms, and host factors determine disease behavior.

Another mechanism of interaction between the virus and infected host is integration of viral DNA into the host genome. While this may cause genomic instability, viral integration is rather thought of as the "chicken and not the egg"—thus integration of viral genomes is a consequence and not the cause of genomic instability. Upon entry into the cervical epithelial cell, the circular HPV genome can be linearized in the E1–E2 genes, which disrupts the E2 open reading frame. In the situation whereby only episomal DNA is present in the cell, E2 protein controls viral replication by suppressing expressing of early genes. Putatively, loss of E2 should increase E6 and E7 expression. But this is not a likely mechanism of viral transformation because the negative feedback loop is abrogated in the simultaneous presence of both episomal and integrated genomes in a cell. Moreover, in high-

grade lesions, aneuploidy and polyploidy can be detected before or in the absence of any viral DNA integration.

16.3.3 Molecular Pathogenesis of CvCa

The uncontrolled proliferation, escape from apoptosis, cellular immortalization, genomic instability, and eventual transformation of HR-HPV-infected cervical epithelial cell are mediated primarily by viral oncoproteins E6 and E7.

Host chromatin structure can be modulated by HPV infection leading to epigenetic alterations. One mechanism of increased *CDKN2A*/*INK4A* expression by E7 is through modification of histone architecture at the *CDKN2A* locus. Moreover, promoter methylation of cell adhesion molecule 1 (CADM1) and T-lymphocyte maturation-associated protein (MAL) is demonstrable in CIN3 lesions and have been explored as potential biomarkers.

The master regulator of cell cycle progression into S-phase, RB, sequesters E2F in a complex and hence prevents its entry into the nucleus to induce expression of genes required for S-phase entry. HR-HPV oncoprotein E7 plays major regulatory roles at this stage of the cell cycle. First, E7 forms complexes with RB, thus releasing E2F to induce genes such as cyclin A and E, which interact with cyclin-dependent kinases to drive S-phase entry. Second, E7 increases the levels of cyclin A and E through binding to their inhibitors, p27 (cyclin A) and p21 (cyclin E). Probably because of the importance of early cell cycle deregulation in malignant transformation, low-grade HR-HPV lesions express high levels of cyclin E.

Viral oncoprotein E7 also increases the expression of *CDKN2A*/*INK4A* and *TP53*. This effect of E7 on *CDKN2A*/*INK4A* should result in cell cycle arrest, because p16 inhibits cyclinD-CDK4/6 complexes, thus preventing hyperphosphorylation of RB to enhance sequestration of E2F. But the effect of or binding of E7 to RB continues to result in the release of E2F independent of RB phosphorylation. Thus, while elevated p16 serves as a biomarker of CvCa, it fails to perform its functions of halting cell cycle progression.

The increased expression of p53 by E7 should maintain genomic stability by its induction of genome repair or apoptosis in cells with irreparable genomic damage. However, the effects of E6 also abrogate this function. Viral oncoprotein E6 forms complexes with p53 and E3 ubiquitin ligase E6-associated protein (E6AP) to ubiquitinate p53 for proteasomal degradation. Apart from loss of p53 apoptotic functions, E6 also prevents cellular apoptosis via other mechanisms. First, E6 can bind to TNFR1, thus interfering with extrinsic apoptosis induction by TNF-α. Second, E6 can bind to proapoptotic BAX and BAK, as well as cause increased levels of apoptotic inhibitors including inhibitors of apoptosis 2 (IAP2) and survivin, thus abrogating the mitochondrial apoptotic pathway as well.

Another mechanism by which HR-HPV infection causes cellular transformation and dysplastic lesion formation is through induction of genomic instability through various mechanisms. First, E6 and E7 oncoproteins can induce aberrant number of

centrosomes during cell division in infected cells. The abnormal number of centrosomes may lead to multipolar mitotic figures with subsequent formation of aneuploidy and polyploidy. Second, the sequestration of p53 by E6 prevents its normal functions of maintaining genomic stability during cell division. Finally, viral genome integration into host genome requires host genome damage by E6 and E7 that could potentially lead to genomic damage.

Maintenance of immortality also relies on reversal or prevention of telomere attrition. In HR-HPV infection, this is partly achieved by increased expression of telomerase reverse transcriptase (*TERT*) to repair shortening telomeres. Additionally, cervical cancers demonstrate gains of chromosome 5p, the *TERT* locus, and 3q that contain the RNA component of telomerase (*TERC*).

16.4 Circulating CvCa Biomarkers

Consistent with many gynecologic cancers, serum proteins including SCCA, CA125, and CYFRA 21-1 are clinically useful CvCa biomarkers. To augment disease management, circulating miRNA, serum proteomics, and circulating CvCa cells are being sought as CvCa biomarkers.

16.4.1 Circulating Cell-Free DNA Content as CvCa Biomarkers

Stage-wise elevation of ccfDNA was demonstrated in CvCa patients [1]. Plasma DNA levels were significantly much lower in CIN3 (8.1 ng/ml) than in stage I CvCa (12.78 ng/ml) patients ($p = 0.001$), and stage II and III levels were much higher (17.99 ng/ml) than stage I ($p = 0.02$). With a diagnostic reference cutoff level at 15.70 ng/ml, a sensitivity, specificity, PPV and NPV were 38.1 %, 92.5 %, 84,21 % and 58.73 % respectively for CvCa detection. Circulating DNA levels in CvCa patients are much higher than in healthy controls [2]. Median plasma DNA was 61.59 mg/l in patients compared to 16.35 mg/l in controls ($p < 0.01$). Similarly, median plasma DNA concentration was lower in stage I (46.02 mg/l) than stage II and III (71.35 mg/l) patients.

A prognostic relevance of DNA methylation in serum samples from CvCa patients has been demonstrated. Hypermethylation of the promoters of *APC* and *RASSF1A* were present in 48 % of plasma samples from patients with high-grade cervical lesions and cancer, compared to 3 % of those with low-grade lesions. The frequency of methylation correlated with FIGO stage [3]. Methylation in any of *CALCA, hTERT, MYOD1, PGR*, and *TIMP3* in serum was positive in 87 % of women with CvCa, and these were present in matched cancer tissue samples [4]. *MYOD1* methylation was associated with advanced stage disease and poor

disease-free survival and OS. Similarly, methylation of *CDH1*/*CDH13* in serum samples was associated with poor DFS [5].

16.4.2 Circulating HPV DNA as CvCa Biomarkers

To study CvCa circulating viral DNA, an assay targeting HPV-insertion sequence was developed. This concept proved useful in detecting specific ctDNA in many CvCa patients, and the levels or amounts reflected tumor burden. In principle, the assay can be used to monitor for residual disease or for detection of subclinical relapse in patients with HPV-positive tumors [6].

HPV DNA has been detected at various frequencies in CvCa patients, and some are associated with clinical parameters. Moreover, viral load appears to increase with disease progression or following relapse. Plasma HPV DNA was positive in 12 % of patients, but those with metastatic disease had higher viral load and were more likely to develop recurrent disease [7]. HPV16, HPV18, and HPV52 DNA were present in blood from 27 % of patients with invasive CvCa, but not in those with CIN or microinvasive disease. Disease recurrence with distant metastasis was associated with positive circulating levels. Similarly, circulating HPV16 DNA was detectable in 30 % of patients with SCC of the cervix, and the copy number was higher in those with invasive cancer compared to patients with CIN3 [8].

Plasma HPV DNA was evaluated for assessing disease progression and response to therapy. HPV16 and HPV18 DNA were detectable in 50 % of cases, and the median level was much higher in patients with cancer and high-grade lesions than those with low-grade pathology. In 79 % of patients, plasma HPV DNA was undetectable following complete response to treatment, but returned to detectable levels in those with recurrences [9]. Widschwendter et al. detected HPV DNA in 45 % of CvCa patients at diagnosis, but these converted to negative following treatment and without recurrences [10]. This serum HPV positivity was present for up to 423 days (median 72 days) prior to clinical diagnosis of relapse

16.4.3 Circulating CvCa miRNA Biomarkers

16.4.3.1 Circulating CvCa Diagnostic miRNA Biomarkers

A number of potential diagnostic circulating miRNA biomarkers for CvCa have been detected. Solexa deep sequencing of pooled serum samples from CvCa patients and healthy controls, followed by library construction and analysis using MIREAP, enabled the identification of two novel miRNAs for CvCa detection [11]. As a single biomarker, one of these achieved a sensitivity, specificity, and AUROCC of 85.7 %, 88.2 %, and 0.921, respectively. Of 12 miRNAs markedly elevated in serum samples from CvCa patients, 5 were validated as CvCa biomarkers [12]. The 5 miRNAs, miR-21, miR-29a, miR-25, miR-200a, and miR-486-

5p, as a panel was more superior at CvCa detection than each marker alone, and outperformed serum SCCA and CA125. A potential association between miR-29a and miR-200a with tumor stage and grade was also suggested. In screening of serum samples from patients with CvCa, CIN, and healthy subjects, miR-16-2*, miR-195, miR-2861, and miR-494 were uncovered as discriminatory diagnostic biomarkers for CvCa [13]. As a signature panel, the four miRNAs yielded an AUROCC of 0.849 and 0.734 in discriminating CvCa from CIN and healthy controls, respectively. While miR-16-2* shares similar circulating profiles as in breast and ovarian cancer patients, the remaining three miRNAs may be unique to CvCa.

16.4.3.2 Circulating CvCa Prognostic miRNA Biomarkers

Circulating miRNAs are associated with clinicopathologic variables and prognosis of CvCa patients as well. Cervical cancer metastasis to lymph nodes is associated with alterations in the levels of miR-20a and miR-203 in lymph node tissues [14]. To evaluate the possible noninvasive utility of these in predicting lymph node metastasis in early-stage disease, pretreatment samples from stage I–IIA patients were obtained for analyses. MiR-20a levels were significantly higher in CvCa patients than controls and were even much significantly higher in those with node metastasis than patients without ($p = 000$, OR, 1.552). Paradoxically, miR-203 levels were significantly higher in CvCa patients than controls, but node metastasis was associated with decreased levels ($p = 0.001$, OR, 0.849). But the levels of miR-20a were more accurate than miR-203 in predicting nodal involvement at a sensitivity, specificity, and AUROCC of 75 %, 72.5 %, and 0.734, respectively. Analyses of both serum and tissue samples from cervical SCC patients and controls uncovered six miRNAs (miR-20a, miR-1246, miR-2392, miR-3147, miR-3162-5p, and miR-4484) that could accurately predict lymph node metastasis with AUROCC of 0.932 for serum samples (sensitivity of 85.6 % and specificity of 85 %) and 0.992 for tissue samples (sensitivity of 96.7 and specificity of 95.0 %) [15]. Although inferior to tissue levels, the serum levels of this signature were superior to SCCA (AUROCC of 0.713) for predicting lymph node metastasis in early-stage SCC of the cervix. Differential circulating levels of miR-141*, miR-646, and miR-542-3p were uncovered between cervical SCC patients and controls [16]. The abnormal postoperative levels of the three miRNAs in patients could serve as biomarkers for treatment monitoring, but were also suitable as diagnostic biomarkers.

A significant decrease in the tumor suppressormir, miR-218, in both tissue and serum samples of patients compared with controls ($p < 0.001$) was uncovered [17]. The decreased levels in cancer patients were associated with aggressive phenotypes as determined by advanced FIGO stage, invasiveness, and lymph node metastasis. MiR-218 has also been investigated for its possible clinical utility in serum samples for CvCa management [18]. There were significant reductions in circulating levels in CvCa patients compared with controls ($p < 0.001$), and the decrease in the levels was associated with adenocarcinoma histology, advanced tumor stage, and lymph node metastasis. A noted elevated level of miR-205 in

CvCa patients is significantly associated with poor tumor differentiation, lymph node metastasis, and advanced tumor stage [19]. The elevated levels could differentiate early from advanced stage disease, metastatic from nonmetastatic disease, and differentiated from poorly differentiated tumors with AUROCC of 0.740, 0,694, and 0.717, respectively. Importantly, Kaplan–Meier analysis associated high levels of miR-205 with shorter OS, and in multivariate Cox regression analysis, miR-205 was an independent prognostic predictor.

16.4.4 Circulating CvCa Protein Biomarkers

Several proteins are overexpressed as a consequence of CvCa progression. These proteins are released into interstitial fluids, such that their levels are subsequently elevated in serum samples from cancer patients. The circulating levels of serum proteins have been investigated for their potential clinical utility. In CvCa, the low sensitivity and the lack of exclusivity of their expression by CvCa cells have made several of these proteins unattractive as screening or early detection biomarkers. However, following diagnosis, they display some potential in patient management in regards to possible disease staging, prognostication, treatment monitoring, and early detection of disease relapse. Of the numerous serum biomarkers including SCCA, CYFRA 21-1, CA125, CA15-3, CEA, IL6, TNF-α, VEGF, hsCRP, IAP, and autoantibodies, SCCA, CA125, and CYFRA 21-1 have been extensively evaluated in CvCa patients (Table 16.1).

16.4.4.1 Serum SCCA as CvCa Biomarker

SCCA is the most extensively evaluated and a potentially promising biomarker for the management of cervical SCC. While in general serum SCCA is not evaluated for diagnostic purposes, an attempt was made to use it as an early detection biomarker of precancerous cervical lesions. Thus, in comparison to CEA and TPA, a lower cutoff value of 0.55 ng/ml for SCCA achieved the best diagnostic sensitivity of 93 % for the detection of CINI–III [20].

Table 16.1 Percentage of CvCa patients with elevated serum proteins at defined cutoff values

Serum protein	Cutoff values	Frequency of elevated levels (%)
SCCA	1.5 ng/ml	88
	2.5 ng/ml	60
CYFRA 21-1	1.0 ng/ml	42
	3.5 ng/ml	52
CA125	16 U/ml	52
	35 U/ml	49

CYFRA21-1; higher frequency in SCC

Serum SCCA and Clinicopathologic Variables of CvCa Patients

Serum levels of SCCA are associated with a number of clinical and pathologic variables of CvCa. The serum levels are elevated in 28–88 % of patients with SCC of the uterine cervix. Although the upper range appears promising for its utility as an early detection biomarker, the variable range due to study design and also use of different cutoff values (1.5–2.5 ng/ml) for disease prediction makes it unattractive for such purposes.

While all squamous cells can express and release SCCA, the circulating levels depend partly on the degree of cellular differentiation and possibly death, because it is released in a passive process. Keratinizing and large cell non-keratinizing CvCa cells release more SCCA than non-keratinizing small CvCa cells. However, various clinicopathologic variables of CvCa are associated with the pretreatment serum levels. These features include FIGO stage, histologic grade, tumor size, depth of cervical stromal invasion, lympho-vascular space status, parametrial involvement, and lymph node metastasis.

In a large cohort of 401 patients, very high levels of SCCA (>10 ng/ml) were associated with lymph node enlargement on CT scans, and SCCA levels were independent predictors of lymph node metastasis in a cohort of 653 women with SCC of the cervix [21, 22]. In CvCa patients inclusive of patients with adenocarcinoma and adeno-squamous carcinomas, the PPV for lymph node invasion increased with increasing levels of serum SCCA. At a cutoff value of 2 ng/ml, the sensitivity and PPV were 58.1 % and 51.4 %, respectively, but the PPV increased to 70 % and 100 % at serum cutoff values of 4 ng/ml and 8.6 ng/ml, respectively [23]. Thus, the risk of lymph node involvement increases with rising SCCA levels. The risk increases 8.4-fold in patients with levels >4 ng/ml compared to patients with lower levels. In stage IB2—IVA cervical SCC patients, the median SCCA level was 6 ng/ml, and increasing levels significantly correlated with para-aortic lymph node involvement ($p = 0.045$) and tumor diameter ($p < 0.05$) [24]. The increasing levels with advancing disease stage may be that metastasis is associated with increasing expression of SCCA in order to overcome apoptosis, and the increasing release could be accounted for by increasing necrotic cell death.

The surgery for stage IB1 patients with parametrial involvement includes the extensive procedure of parametrectomy. Thus, prior staging knowledge helps with surgical planning. In a series of 115 patients with 15.7 % diagnosed with parametrial disease spread, MRI-based tumor diameter of $\geq$2.5 cm (OR, 9.9), tumor volume $\geq$5000 mm^3 (OR, 13.3), as well as serum SCCA $\geq$ 1.5 ng/ml and CA125 $\geq$ 35 U/ml (OR, 5.7) were independent risk factors for parametrial involvement in multivariate analysis [25].

Serum SCCA as Prognostic Biomarker of CvCa

Whereas many studies report the association of elevated pretreatment serum SCCA levels with prognostic indices, a few have failed to observe these associations. The

risk of death increased in patients with levels >4.5 ng/ml compared to those with ≤1.3 ng/ml, and pretreatment levels were significantly predictive of PFS ($p < 0.026$) and OS ($p < 0.001$) in univariate analyses [26, 27]. In the large cohort (779) analyses by Yuan et al., of stage Ib–IIb patients, elevated pretreatment levels were equally associated with poor prognosis in univariate (but not multivariate) analyses [28]. In patients who received radiotherapy, pretreatment levels >10 ng/ml were significantly associated with worse outcome in multivariate analysis [21]. Strauss et al., observed that even levels >3 ng/ml were independent predictors of recurrence-free survival and OS in stage Ia_2–IIb SCC patients treated by radical hysterectomy [29].

SCCA may still be useful for prognostication under certain circumstances. For example, in 75 patients with recurrent disease in whom SCCA levels were increased, an association with shorter post-recurrence survival was realized, and this was an independent prognostic factor in multivariate analysis. In this cohort of patients, SCCA levels <14.0 ng/ml were associated with better survival in those treated with radiotherapy or surgery than those treated with chemotherapy or supportive care. Patients with levels ≥14.0 ng/ml did not benefit from salvage therapy [30]. Also in stage IB1–IIB cervical SCC patients who received neoadjuvant chemotherapy, decreased pretreatment and posttreatment SCCA ≤3.5 ng/ml was associated with better 3 year DFS and OS. In multivariate analysis, posttreatment levels were strong independent predictor of OS ($p = 001$) and DFS ($p = 0.012$).

Serum SCCA as Treatment Monitoring Biomarker of CvCa

Circulating levels of SCCA may potentially predict response to various treatment regimens of CvCa patients. In patients with locally advanced CvCa, SCCA levels >5 ng/ml were associated with poor response to chemotherapy in multivariate analysis [31]. Moreover, pretreatment levels predicted response to neoadjuvant chemotherapy in patients with advanced stage disease. Levels also decreased in a majority of patients after treatment. In 72 patients, 60 % had pretreatment levels of ≥2.8 ng/ml, but following radiotherapy treatment, only 2 % had serum SCCA levels above this level in the complete response group, and 13 % of those who achieved partial remission [32]. At 2–3 months follow-up after radiotherapy, persistently elevated levels were strong predictor of treatment failure in association with the presence of distant metastasis [21]. Serum SCCA normalization following radiotherapy or chemoradiotherapy was associated with better recurrence-free survival as evidenced by the 74.3 % vs. 5.65 survival in patients with normalized vs. persistent levels [33].

Serum SCCA as Recurrent Monitoring Biomarker of CvCa

Consistent with other circulating biomarkers, and in other cancers, elevated serum SCCA may precede clinical diagnosis of CvCa relapse. Depending on study design

and cutoff values used for detection, 46–92 % of patients have elevated levels prior to clinical detection of recurrence, with a lead time of 2–7.8 months. Thus, rising serum SCCA levels have been detected with a sensitivity of 80 % in CvCa patients with locoregional or distant failures. But, whether early detection of recurrence reduces mortality was questioned. However, ~ 50 % of patients with recurrent disease detected by serial SCCA measurements were good candidates for curative-intent salvage therapy [34].

Because SCCA can be released by other cells and not only CvCa cells, a conundrum exists as to how to manage patients who have been successfully treated, but on follow-up demonstrate isolated elevations of SCCA without symptoms or findings on imaging. In such patient population, FDG-PET is a useful investigational tool. For example, in the series by Chang et al., of patients who had complete response but later presented with isolated increases in SCCA > 2.0 ng/ml on two consecutive occasions, FDG-PET was positive in 94 % of those with recurrent diseases [35]. These patients received curative-intent treatment with good outcomes. The sensitivity and specificity of PET for recurrence detection were 91.5 % and 57.1 %, respectively. But lung metastasis <1 cm^3 were more difficult to detect, and benign lesions such as fibrosis gave false-positive signals. In multivariable comparison of nine CvCa serum biomarkers, only SCCA and hsCRP achieved optimal AUROCCs of 0.822 and 0.831, respectively, for recurrence prediction. The performance was enhanced to AUROCC of 0.870 for a combined model. As a risk predictor, each unit rise in SCCA (ng/ml) and hsCRP (mg/ml) was associated with disease recurrence, with OR of 1.227 for SCCA and 1.025 for hsCRP [36].

16.4.4.2 Serum CA125 as CvCa Biomarker

Serum CA125 and Clinicopathologic Variables of CvCa Patients

CA125 is more of a biomarker for cervical adenocarcinoma than SCC. Serum CA125 levels are elevated in 20–75 % of patients with cervical adenocarcinoma. The levels are also elevated in some cases of cervical SCC, but at much lower frequencies. For instance in one series, 75 % of patients with adenocarcinomas had elevated CA125 levels compared to only 26 % of SCC patients [37]. Consistent with SCCA, the elevated levels of CA125 correlate with tumor stage, histologic grade, tumor size, lymphovascular space involvement, depth of cervical stromal invasion, and lymph node metastasis. In patients with stage Ib–IIa cervical adenocarcinoma, median preoperative CA125 of 34 U/ml was significantly associated with lymph node invasion, which contrasted with node-negative patients with median levels of 17.6 U/ml [38]. In the node-negative patients, the use of logistic regression identified CA125 levels >26 U/ml to be significantly associated with lymphovascular space involvement ($p = 0.04$) and deep stromal invasion ($p = 0.002$).

Serum CA125 as CvCa Prognostic Biomarker

The use of pretreatment CA125 in prognostic predictions has been evaluated with some potential clinical value. In Cox regression analysis, serum CA125 and SCCA were the most important prognostic factors and were independent predictors of survival [39]. CA125 was of prognostic value in multivariate analysis of women with cervical adenocarcinomas [40]. In women with locally advanced cancer who received neoadjuvant chemotherapy and radical surgery, CA125 levels were independent predictors of survival [41], and in those who received radiotherapy, pretreatment levels predicted outcome in multivariate analysis [42]. Thus, pretreatment circulating levels of CA125 are associated with survival outcomes in women with cervical adenocarcinomas.

Serum CA125 as CvCa Recurrence Monitoring Biomarker

In multiple studies, elevated CA125 during follow-up has been associated with recurrent adenocarcinoma of the cervix. In a multi-marker study consisting of CA125, CEA, and SCCA, 74 % of patients with elevations in at least one biomarker within 3 months after treatment had a residual disease or subsequently relapsed, compared to just 15 % of those with normal levels [43].

16.4.4.3 Serum CYFRA 21-1 as CvCa Biomarker

Compared to SCCA, CYFRA 21-1 is released mostly by non-keratinizing CvCa cells.

Serum CYFRA 21-1 and Clinicopathologic Variables of CvCa Patients

With cutoff values in the range of 1.06–3.5 ng/ml, elevated levels have been demonstrated in 42–63 % of patients with SCC of the uterine cervix. Similar to SCCA and CA125, elevated pretreatment serum levels of CYFRA 21-1 are associated with tumor stage, size, stromal invasion, lymphovascular space status, and lymph node metastasis. While levels are higher in advanced than early-stage disease, CYFRA 21-1 is a less sensitive biomarker than SCCA for early and advanced stage CvCa. However, the increased circulating levels of CYFRA 21-1 are significantly associated with lymphovascular space involvement and lymph node invasion on logistic regression analysis [44, 45]. Similar to SCCA, CYFRA 21-1 levels are associated with parametrial invasion. Different biomarkers may predict parametrial involvement in pre- and post-menopausal women with stage 1B CvCa. While tumor size $>$4 cm (OR, 10.029) and serum SCCA $\geq$3.60 ng/ml (OR, 4.132) predicted parametrial invasion in premenopausal women, the predictive factors in postmenopausal women were tumor size $>$3 cm (OR, 11.353) and serum CYFRA 21-1 $\geq$ 2.40 ng/ml (OR, 8.048) [46].

Serum CYFRA 21-1 as CvCa Prognostic Biomarker

The prognostic relevance of serum CYFRA 21-1 is questionable because of lack of strong evidence. In one series, there was a significant association with both DFS ($p = 0.002$) and OS ($p = 0.005$) in univariate analysis of stage Ib–IIa SCC patients. But this was not achieved in Cox regression analysis [27]. In another study of 119 patients inclusive of both adenocarcinoma and adeno-squamous carcinoma, pretreatment levels were significantly associated with disease-free survival and OS, but again this was only in univariate analysis [47]. CYFRA 21-1 and SCCA serum levels correlated with clinicopathologic factors. However, in Cox regression analysis, tumor size >4 cm was an independent predictor of DFS (OR, 3.11) and OS (OR, 3.497) in patients with cervical SCC. Pretreatment levels of CYFRA 21-1 were the only independent factors for DFS and OS in patients with non-SCC [48].

Serum CYFRA 21-1 as CvCa Treatment Monitoring Biomarker

Increased CYFRA 21-1 levels may have predictive potential in CvCa patients. In the large series of 114 stage Ib–IV patients treated with radiotherapy or concurrent chemo-radiation, incomplete treatment indicative of the presence of residual tumor was associated with elevated CYFRA 21-1 levels in 70 % of the cases. Additionally, patients with normal levels post-therapy experienced better DFS than those who failed to achieve normalized levels [49].

Serum CYFRA 21-1 as CvCa Recurrence Monitoring Biomarker

Similar to SCCA, post-therapy increase in CYFRA 21-1 levels during follow-up may precede clinical detection of disease progression in 61.5–89.5 % of patients, with a lead time of 2–4 months. In the study by Kainz et al., 61.5 % of patients with local recurrences had elevated CYFRA 21-1 levels [50]. Because CYFRA 21-1 is less sensitive than SCCA at recurrence detection, its combination with SCCA enabled a mean lead time of 5.25 months to be achieved in patients with recurrent disease. But the comparison of the two biomarkers by Callet et al. reveals identical performances at recurrence detection [51]. The sensitivity, specificity, PPV, and NPV were 89.5 %, 86.4 %, 91.9 %, and 92.7 %, respectively, for CYFRA 21-1 and 75 %, 99.1 %, 98.3 %, and 85.2 %, respectively, for SCCA, with median lead times of 60 days for CYFRA 21-1 and 50 days for SCCA.

16.4.4.4 Serum VEGF as CvCa Biomarker

Elevated levels of VEGF isoforms occur with progressive or persistent disease and may potentially be useful in treatment response prediction and prognostication in

women with CvCa. VEGFC, VEGFD, and VEGFR3 are expressed in >50 % of CIN3 lesions. Serum levels are also elevated in CvCa patients at diagnosis and have decreased on successful therapy. Elevated levels >4436 pg/ml are detected in 0 %, 10 %, 20–27 %, 57 %, and 60 % of patients diagnosed with ASCUS/normal histology, ASCUS/CIN1, CIN2–3/early-stage CvCa, advanced stage CvCa, and those with persistent disease, respectively [52]. Serum VEGFB levels >865 pg/ml were observed at slightly much higher frequencies of disease progression (11 % of ASCUS/CIN1, 24 % of CIN1, 43 % of CIN2–3, and 60–70 % of CvCa patients and those with persistent disease). Patients on remission following radiotherapy or chemo-radiation had normal circulating VEGFB and VEGFC levels. VEGF levels >244 pg/ml were associated with 5 months median time to progression compared to 19 months for those with lower levels [53]. In another series of patients undergoing radio-chemotherapy, disease recurrence was predicted by elevated serum VEGFA before and after treatment. A rise in VEGFA >500 pg/ml as well as a decrease (>9 %) in pretreatment TIMP2 levels were significant predictors of early recurrence with RR of 8.5 and 11.0, respectively. These parameters were independent prognostic factors for OS [54]. Median pretreatment VEGF level was 647.15 pg/ml; however, significantly elevated levels were associated with women <50 who had stage IIIa–IVa tumors >4 cm, with lymph node metastasis. In logistic regression analysis, pretreatment serum VEGF levels and tumor size were independent risk factors for treatment outcome. Elevated levels conferred worse prognosis in patients with tumor size >4 cm [55].

16.4.4.5 Serum IAP as CvCa Biomarker

Circulating levels of immunosuppressive acidic protein (IAP) are elevated in CvCa patients. Increased serum levels were in 43 % of CvCa patients, and this was associated with advanced stage and tumor size [56]. Pretreatment levels >620 ng/ml were demonstrable in as many as 78 % of patients with advanced cervical SCC. Moreover, elevated levels significantly correlate with tumor size. Increased serum levels were in 81 % of patients with tumor size >5 cm compared to 44 % of those with small tumors ($p = 0.007$). Pretreatment increase in IAP level was an independent predictor of poor PFS (RR, 3.009) and OS (RR, 3.436) [57]. Disease recurrence may also be predicable by elevated IAP levels. Serum levels were elevated in all patients with recurrent disease compared to 17 % of those free of relapse [56]. Elevated IAP (>613 μg/ml) was demonstrated in 50.7 % of CvCa patients, while the corresponding frequency for SCCA (> 2.5 ng/ml) was 73 %, and the two biomarkers showed significant correlation. The elevated levels of IAP were associated with lymph node metastasis and shorter survival. In multivariate analysis, serum IAP was a significant predictor of poor survival alone ($p = 0.049$), in conjunction with lymph node involvement ($p = 0.007$), or advanced stage disease ($p = 0.008$) [58].

16.4.4.6 Other Potential Serum Protein Biomarkers of CvCa

Other potential serum biomarkers have been studied for CvCa management. In cervical SCC patients, serum high mobility group box chromosomal protein 1 (HMGB1) achieved the best diagnostic accuracy for recurrent disease with AUROCC of 0.816 compared with 0.768, 0.703, and 0.625 for SCCA, CYFRA 21-1, and CEA, respectively. While specificity (78 %) and PLR (3.25) were best for HMGB1, SCCA achieved the best sensitivity (76.3 %) and NLR (0.34) [59]. In another study, analysis of 22 serum biomarkers prior to treatment of CvCa patients uncovered CA15-3, SCCA, and TNF-α to be associated with prognosis. However, in Cox multivariate analysis and classification, together with regression tree analysis, 2-year survival was significantly better for patients with low levels of CA15-3 (<17.6 μg/ml) and TNF-α (<10.6 pg/ml) [60].

16.4.4.7 Circulating CvCa Proteomic Biomarkers

Serum and plasma proteomics for identification of clinically useful biomarkers for CvCa have been explored. Both discriminatory peaks and potentially useful diagnostic proteins enabled accurate detection of cervical lesions and cancer. The use of cation-exchange protein chips and SELDI-TOF MS enabled identification of 6 plasma peaks that could differentiate in situ and invasive CvCa from control samples at sensitivity of 91 % and specificity of 97 % [61]. Indeed, two peaks at Mr 6586.41 and 3805.68 achieved similar discriminatory ability with sensitivity and specificity of 92 % and 97 %, respectively. Three other serum protein peaks at *m*/*z* 3974 Da, 4175 Da, and 5906 Da were also identified through MALDI-TOF MS for CvCa detection [62]. The discriminatory performance of the peaks between CvCa and healthy controls achieved a sensitivity of 87.5 % and specificity of 90 % in the test data set. Matthews et al. uncovered two protein peaks by MALDI-TOF MS that could differentiate higher-grade CIN patients from controls [63]. A peak at *m*/*z* 4459 was associated with high risk for high-grade CIN, yielding a sensitivity of 58 % for CIN2 and 75 % for CIN3 lesions. However, increasing intensity of a second peak at *m*/*z* 4154 was associated with higher risk in Caucasian-American and a rather lower risk in African-American women. Guo et al. used 2D DIGE followed by MALDI-TOF MS and Ingenuity Pathway Analysis software to identify ten plasma proteins for cervical SCC [64]. These included proteins related to metabolism (APOAI, APOA4, APOE), complement (EPPK1, CFHR1), enzymes (CP, FZ, MASP2), glycoprotein (CLU), and immune functions (IGK@-immunoglobulin kappa locus). Further validation of APOA1, APOE, and CLU were performed with plasma from women with cervical lesions at different stages. Another application of 2D gel electrophoresis followed by protein identification by MS uncovered differential plasma levels of 18 proteins between CvCa patients and controls [65]. However, ELISA was used to validate two peaks, identified as CK19 and tetranectin. In a follow-up study, serum protein separation by 2D DIGE followed by identification by tandem MS uncovered 20 differentially expressed

proteins between cancer and controls [66]. Four of these proteins, complement factor H, CD5-like antigen, gelsolin, and ceruloplasmin were validated. Boichenko et al. used iTRAQ, label-free shutgun, and targeted MS quantification to identify serum proteins for CIN and CvCa [67]. Six elevated proteins, α-1-acidic glycoprotein 1, α-1-antitrypsin, serotransferrin, haptoglobin, α-2-HS-glycoprotein, and vitamin D-binding protein, were validated with UHPLC/MRM on separate serum samples. As a panel, the 6 biomarkers yielded a sensitivity of 67 % and specificity of 88 % for discriminating between CIN and healthy control samples.

16.4.4.8 HPV Serology in CvCa

Serologic assays targeting high-risk HPV detection, as a screening test has been sort for sometime. These anti-HPV serologic tests are currently at the research stage, although they hold great potential for screening of women in underserved communities, where routine Pap smear screening programs are ineffective. Despite the inherent issues with test accuracies, the performance of serum anti-HPV antibodies has been evaluated [68]. Of the three HPV antigens, (E4, E7, and VLPs-L1), seropositivity rates among women with CvCa were high for E4 (73 %) and E7 (80 %). The presence of 1, 2, or 3 of these antibodies elevated the odds for CvCa, with ORs of 12.6, 19.9, and 58.5, respectively. The best performing antigens, E4 and E7, as a panel achieved a sensitivity of 93.3 % and specificity of 64.1 % for discriminating between CvCa and CIN2 and three lesions.

16.4.5 Circulating CvCa Cells

DTCs are associated with pathologic features of CvCa, while circulating cervical cancer cell (CCvCaC) characterization, though in its infancy, may in future be useful in disease management. Using A45B/B3 antibody, DTCs were detected in 26% of CvCa patients (54 patients). DTC-positivity was however associated with FIGO stage, grade, and lymph node status [69]. In an expanded cohort of 102 patients, the detection rate of DTC was 19 %, and the presence of DTCs was associated with the previous pathologic parameters in addition to tumor size [70]. In another large study (228 patients), the detection of DTCs was 16% [71]. The association with pathologic features remained, with additional association with lymphangiosis. In multivariate analysis, the only predictor of DTC presence was tumor size.

CCvCaCs have also been detected in CvCa patients, although their rate of detection and clinical relevance await evaluation. A sensitive digital RT-PCR targeting HPV-oncogene transcripts enabled direct analysis and detection of CCvCaCs [72]. In a pilot study, 3/10 CvCa patients were positive for CCvCaCs. In one patient who suffered lung tumor cell emboli, markedly elevated CCvCaCs were in the patient blood [73].

16.5 Summary

- CvCa is the fourth most common female cancer, with high disease prevalence due to intensive screening for early detection.
- Unfortunately, the majority (~85 %) of CvCas are diagnosed in middle- to low-income countries, where the mortality is equally high.
- The primary etiologic agent of CvCa is high-risk HPV infection of the uterine cervix.
- The molecular pathology of CvCa therefore is primarily deregulation of oncogenic signaling pathways by HPV oncoproteins.
- Established circulating biomarkers for CvCa management include SCCA, CA125, and CYFRA 21-1.
- Circulating miRNAs are emerging as potential noninvasive CvCa biomarkers.
- Serum proteomics and circulating CvCa cell characterization are still at their infancy.

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Chapter 17
Endocrine Cancer Biomarkers in Circulation

Key Topics

- Molecular pathology of endocrine cancers.
- Circulating thyroid cancer (ThyCa) epigenetic, genetic, and transcript biomarkers.
- Serum proteins (antithyroglobulin antibodies) as ThyCa biomarkers.
- Circulating ThyCa cells.
- Circulating endothelial cells as ThyCa biomarkers.
- Circulating pheochromocytoma (PCC) and paraganglioma (PGL) biomarkers.

Key Points

- The most common malignancy of the endocrine system is ThyCa, followed by those of the adrenal gland. ThyCa mortality is globally low; however, this remains high in the developing parts of the world.
- Circulating thyroid cancer biomarkers of clinical relevance include ctDNA detection by gene methylation and mutations (e.g., $BRAF^{V600E}$), as well as transcripts and antibodies to thyroglobulin (TG). Circulating miRNA, proteomic discoveries, circulating ThyCa, and endothelial cells are emerging biomarkers.
- There is paucity of circulating biomarkers for adrenal cortical tumors, but PCC and PGL are managed by monitoring the levels of serum catecholamines and granins.

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17.1 Introduction

The endocrine system is a multi-tissue and organ system that regulates almost all body functions through secretions (hormones) that are spread throughout the body via the vasculature. They are anatomically organized into connective-tissue delineated cells arranged in clumps, cords, or follicles, as well as diffused cells in organs such as the kidney, heart, gastrointestinal system, and reproductive organs. In regard to malignant transformation, the common malignancies are associated with the well-organized tissues, especially the thyroid and adrenal glands, with low incidence cancers among the remaining endocrine structures.

Neoplastic transformation of thyroid follicular cells is the most common malignancy of the endocrine system. Although not as common as other epithelial cancers, the estimated 2012 global ThyCa incidence was 68,179, with 56.5 % occurring in the less developed world. In the same year, the estimated mortality was 12,626, but unlike the incidence distribution, the deaths in the less developed world (71.1 %) more than doubled those of the developed world. Thus, while in the US 64,300 new cases are expected in 2016, the projected mortality is only 1,980. Most thyroid nodules are benign follicular adenomas. Only ~5 % of thyroid tumors are malignant, mainly carcinomas of follicular cells. ThyCas are three times more prevalent in women than men, and the global incidence has been on the rise over the past few decades, probably as a result of improved early detection of possibly nonprogressive tumors.

ThyCas are histologically classified into three main groups, namely well-differentiated thyroid carcinomas (papillary and follicular carcinomas), poorly differentiated thyroid carcinomas, and undifferentiated thyroid carcinomas or anaplastic ThyCas. Parafollicular or C cell tumors are referred to as medullary ThyCas. Well-differentiated ThyCas have good prognosis, with 10-year survival of 80–95 %. However, recurrences can occur 30 years after diagnosis even in those supposedly deemed to have been cured. Biomarkers that can be used for noninvasive monitoring of recurrence will thus be useful for follow-up surveillance. Anaplastic ThyCa is the most aggressive subtype, being responsible for >30 % of all ThyCa-related deaths. Biomarkers for the early detection and treatment monitoring of ATC have thus been explored.

Tumors of the adrenal medulla and cortex are also rare. Many (80–85 %) chromaffin cell tumors arise from the adrenal medulla and are referred to as pheochromocytomas. The remaining tumors originate in the extra-adrenal sympathetic and parasympathetic nervous system and constitute the paragangliomas. These tumors are rare with incidence of 2–5 per million per year. They are mostly benign, with malignancy occurring in 10–15 % of all cases. Thus, the overall survival is good at ~89 %, but when malignant, the survival outcome is dismal at 20–70 %. It is therefore imperative to establish malignancy at the earliest possible time during the diagnostic work-up.

Carcinomas from the adrenal cortex are equally rare, with estimated annual incidences of 0.7–2 per million. They are more common in children <5 and adults between ages 40–50. Because of inefficient therapies, complete surgical tumor resection remains the mainstay of curative treatment. Thus, recurrences are

common, occurring in 60–80 % of cases. Patients who develop recurrent disease and those initially diagnosed with advanced stage IV disease have 5-year survival rate of <5 %. Indeed, those unsuitable for resection only survive 3–9 months. There is thus a needed understanding of the molecular pathology and development of effective therapies for adrenal cortical tumors. Noninvasive biomarker assays for early detection and disease management are of importance as well. However, the rarity of these tumors may pose as a challenge to the development of effective novel therapies, but he effort is worthwhile.

17.2 Screening Recommendations for Endocrine Cancer

Because of the low incidence and prevalence of endocrine cancers, screening is not recommended for the general population at moderate to low risk. However, certain risk factors elevate the probability of people developing certain types of cancers. For example, the risk factors for ThyCa include gender, age, hereditary factors, radiation exposure, and low iodine intake. Female gender is associated with elevated ThyCa risk and early age at diagnosis (40–50 years compared to 60–70 years in men), while radiation exposure to the head and neck region, especially during childhood and adolescence, increases the risk. Family history of ThyCa, genetic risk factors such as inherited *RET* mutations, and diet low in iodine are all associated with elevated risks above the general population. Therefore, people with these risk factors are periodically examined for early detection of ThyCas. The surveillance work-up includes physical examination of the thyroid and cervical lymph nodes, imaging especially by diagnostic ultrasound and serum measurement of thyroid-stimulating hormone. Normal or elevated TSH carries very low risk for cancer; however, low levels require radionuclide thyroid scan. Again a “hot” or autonomous nodule carries low risk for cancer.

17.3 Molecular Pathology of Endocrine Cancers

The molecular pathology of cancers of the thyroid and adrenal glands will be presented followed by synopsis of other endocrine glands and tissues.

17.3.1 Molecular Pathology of ThyCa

The thyroid gland is composed primarily of the thyroxin-secreting follicular cells. In addition, there are scattered parafollicular or “C” cells that secrete calcitonin. As a result, the majority of ThyCas originate from these two cell types. ThyCas are heterogeneous in nature because any cell type in the gland can give rise to tumors. Thus, both thyroid epithelial and non-epithelial cells are susceptible to

transformation. However, almost all thyroid tumors are of epithelial cell origin. Based on the cell of origin, primary thyroid tumors fall into two categories. Many (>95 %) ThyCas are of follicular cell origin, with just 2–5 % emanating from parafollicular cells, and rarely from other non-epithelial cell types in the thyroid. Tumors of follicular cell origin can be benign (follicular adenomas and oncocytomas) or malignant. The malignant cancers are further subclassified into papillary ThyCa (PTC), follicular ThyCa (FTC), poorly differentiated ThyCa, and undifferentiated or anaplastic ThyCa (ATC). Papillary ThyCa, FTC, benign thyroid tumors, as well as the normal follicular cells can acquire genetic lesions and progress to the poorly differentiated and anaplastic carcinomas. Noteworthy, there are several other minor variants of ThyCas such as epithelial tumors of uncertain origin, mixed follicular and C cell tumors, as well as the other subtypes of non-epithelial cell origin.

The molecular pathology of ThyCa is well elucidated. Mutations in genes that primarily control the MAPK pathway are strongly implicated in thyroid carcinogenesis. Mutually exclusive mutations in *BRAF, RAS*, and *RET/PTC* rearrangement underlie the vast majority (>70 %) of PTC. Similarly, >70 % of FTC is characterized by mutually exclusive mutations in *RAS* and rearrangement mutations in *PAX8/PPARγ*. While ATCs harbor some of these mutations (e.g., ~25 % have *BRAF* mutations), they are characterized by additional mutations involved in the WNT/β-catenin, PI3K, and cell cycle pathways. Activating mutations in *CTNNB1* that lead to WNT/β-catenin signaling are found in 25–60 % of ATC. Similarly, loss of function in *PTEN* tumor suppressor leading to PI3K pathway activation is present in up to 16 % of ATCs. Over 50 % of ATCs harbor *TP53* mutations, while mutations in the promoter region of *TERT* are demonstrated in 30–50 % of these tumors. *TERT* mutations are frequently found in tumors with *RAS* or *BRAF* mutations.

17.3.1.1 *BRAF* Mutations in ThyCa

Activating mutations in *BRAF* are demonstrable in about 35–70 % of PTCs. Whereas mutations have been described at codons 598, 599, 600, and 601, the *BRAF* valine substitution with glutamic acid at nucleotide position 1799 of codon 600 is the most common, occurring in up to 90 % of PTCs. This mutation is more common in the tall cell variant of PTC and is associated with aggressive disease and relapse, and hence is a predictor of poor prognosis. PTC originating on the background of radiation exposure may harbor *AKAP9/BRAF* rearrangements.

17.3.1.2 *RAS* Mutations in ThyCa

The RAS genes (*KRAS, NRAS*, and *HRAS*) are mutated in ThyCa. While *KRAS, NRAS*, and *HRAS* codon 61 mutations have been reported, *KRAS* codon 12 and 13 mutations are the most common. These mutations are present in 20–40 % of adenomas, in 10–15 % of PTCs (especially the follicular subtype), and in 40–50 %

of FTCs. However, they are detected in ~35 % of poorly differentiated thyroid carcinoma and up to 50 % of ATCs.

17.3.1.3 *PIK3CA* Mutations in ThyCa

Mutations in this gene are present in 25–45 % of ThyCas. The mutations, which are commonly in exons 9 and 20, are rare in PTC (just up to 5 %) but more common in poorly differentiated and anaplastic cancers. Moreover, the *PIK3CA* genomic locus, 3q26.3, is amplified in ~40 % of ATCs.

17.3.1.4 *PAX8/PPARγ* Rearrangement in ThyCa

Translocation between chromosomes 2 and 3 t(2;3)(q13;p25) gives rise to the fusion of the thyroid-specific paired domain transcription factor, *PAX8*, with *PPARγ* giving rise to the *PAX8/PPARγ* rearrangement. This causes overexpression of *PPARγ* in ThyCas. This gene rearrangement, which tends to be exclusive of *RAS* mutation, is common in FTC, with a frequency of 30–40 %, but is associated with favorable prognosis. These genomic rearrangements are also found in a small fraction of benign thyroid tumors. About 2–10 % of follicular adenomas and ~5 % of oncocytomas harbor them. Rarely (<5 %), the *PAX8/PPARγ* rearrangement has been reported in the follicular subtype of PTC.

17.3.1.5 *RET/PTC* Rearrangement in ThyCa

The fusion of the 3′ end of *RET* tyrosine kinase receptor to the 5′ ends of a number of genes creates the *RET/PTC* rearrangements. *RET/PTC1* and *RET/PTC3* are intra-chromosome 10 fusions, while the rest including *RET/PTC2* are fusions of genes between chromosomes. However, all these fusion genes retain the tyrosine kinase activity of RET and hence activate the MAPK pathway important in thyroid carcinogenesis. These rearrangements are demonstrable in 10–20 % of sporadic adult PTCs but are clustered more in patients with a history of radiation exposure where as many as 50–80 % will have them. In sporadic non-radiation-mediated PTC, *RET/PTC1* (frequency of 50–70 %) and *RET/PTC3* (frequency of 20–30 %) are the most common.

17.3.2 Molecular Pathology of Adrenal Cortical Cancer

Adrenal cortical tumors (ACTs) are diseases of epigenetics and genetics. While the molecular pathology is not fully unraveled, some progress is made in the alterations of genes important in the development and progression of this disease. Specific

gene methylations, miRNA deregulation, mutations in specific genes, and deranged growth factor signaling have all been demonstrated in adrenocortical cancers (ACCs).

17.3.2.1 Epigenetics of ACTs

DNA methylation and miRNA deregulation underlie ACCs. ACCs demonstrate global genomic hypomethylation compared with benign adrenal tumors and normal adrenal tissues. However, promoter hypermethylation at CpG islands occurs in a number of genes including *NTNG2, KIRREL, KCTD12, SYNGR1, GATA4, CDKN2A, DLEC1, PYCARD,* and *SCGB3A1*. Of interest, promoter methylation at 11p15, the *IGF2* and *H19* imprinted gene locus, is found in ACCs.

There are differential miRNA expressions between ACC and adrenal cortical adenomas (ACAs) and normal adrenal tissues. MiR-139-5p, miR-184, miR-210, miR-483-5p, and miR-503 are upregulated, while miR-139-3p, miR-195, miR-335, and miR-675 are downregulated in ACC compared to control samples. Of importance, miR-483-5p and miR-195 are reproducible. MiR-483-5p may control *IGF2* overexpression and the levels are potential diagnostic and prognostic biomarkers. The potential of circulating miR-483-5p and miR-195 in management of patients with ACC has been demonstrated.

17.3.2.2 Chromosomal Changes in ACTs

Alterations in chromosomal regions that harbor genes mutated or underexpressed in ACC characterize these tumors as well. LOH and CGH studies indicate increased frequency of chromosomal losses and genetic lesions in sporadic ACC, and the frequencies are much higher in these cancers compared to adenomas. The regions of chromosomal losses include 2p16, 11q13, 11p15, 17p13, and 17q22–24. These loci house important genes, such as *TP53* (17p13) involved in the pathogenesis of ACC.

17.3.2.3 *TP53* Mutations in ACTs

Germline mutations in *TP53* characterize the vast majority (50–80 %) of childhood ACCs, while somatic mutational inactivation is found in 20–35 % of sporadic adult ACCs. In sporadic adult ACC, hotspot exon 5–8 mutations occur in 20–27 % of the cases. LOH at the *TP53* locus occurs in ~80 % of ACCs. However, these do not match *TP53* mutations or loss of function, suggestive of other pertinent genes in this locus. Candidate genes include *ALOX15B* and *ACADVL*, which have decreased expression in ACCs. The importance of *TP53* mutations in ACC is testified by the disproportionately high incidence of ACCs in southern Brazil. Here, the recurrent

TP53 germline mutation, p.R337H, accounts for the majority of childhood cases. Approximately, 80% of all sporadic ACCs harbor this mutation in southern Brazil.

17.3.2.4 Growth Factor Signaling Pathways in ACTs

Growth factor signaling, primarily through the PI3K and RAS-MAPK pathways, is demonstrated in many ACCs. Abnormal expressions of VEGF, TGFα, TGFβ1, βFGF, and cytokines characterize ACC. VEGF and its receptor, VEGFR2, is overexpressed in 70–80 % of ACCs. Indeed, circulating VEGF levels in patients with ACC fall following tumor removal. Over 90 % of ACCs overexpress EGFR, and FGFR1 and FGFR4 levels are high in ACTs.

17.3.2.5 *IGF2* Alterations in ACTs

The *IGF2* locus, 11p15, shows LOH in ~80 % of ACCs. This locus also contains *H19* and *CDKN1C/p57kip2*. Abnormal imprinting, such as duplication of the paternal allele and loss of the maternal allele (which houses the H19 gene), leads to overexpression of *IGF2*. Together with other regulatory modes, as many as 80–90 % of ACCs overexpress the *IGF2* gene.

17.3.2.6 *SF1* Alterations in ACTs

The amplification and overexpression of steroidogenic factor 1 (*SF1*) occurs in a substantial number of both childhood and adult ACCs. However, these abnormalities are more frequent in pediatric than adult ACCs. Overexpression of *SF1*, which occurs in some tumors without amplification, suggests alternative regulatory pathways of *SF1* overexpression in ACCs.

17.3.2.7 WNT/β-Catenin Pathway Alterations in ACTs

Intracellular accumulation of β-catenin and WNT/β-catenin pathway activation occurs in both benign (38 %) and malignant (>70 %) ACTs. While not too clear, somatic mutations in β-catenin, which occur in ~27 % of ACA and in 31 % of ACC, may partly account for the lack of β-catenin degradation.

17.3.2.8 *MC2R* Alterations in ACTs

LOH and hence inactivation of the ACTH receptor (*MC2R*) is associated with a subset of ACTs. However, in patients with functional tumors, MC2R shows a rather

upregulated expression. The loss of MC2R causes decreases in mineralocorticoid and glucocorticoid production as well as adrenocortical atrophy.

17.3.3 Molecular Pathology of Pheochromocytoma and Paraganglioma

The vast majority (up to 85 %) of chromaffin cell tumors arise from the adrenal medullary chromaffin cells (referred to as pheochromocytomas—PCCs). The remainder is derived from extra-adrenal sympathetic nervous system of the chest, abdomen, and pelvis as well as the head and neck parasympathetic nervous tissues (known as paragangliomas—PGLs). Whereas tumors from sympathetic tissues are endocrinologically active, those from the parasympathetic system are silent and hence cannot be detected using the standard biochemical assays targeting circulating catecholamine.

Although identical, the molecular pathology of PCCs and PGLs is very diverse. Germline and somatic gene mutations account for ~52 % of all cases. Germline heritable mutations in ten genes account for ~35 % of all cases. Thus, PCC and PGL syndromes are accounted for by mutations in the von Hippel Lindau (*VHL*), multiple endocrine neoplasia 2 (*MEN2*/*RET*), the four succinate dehydrogenase genes (*SDHA*, *SDHB*, *SHDC*, and *SDHD*), neurofibromatosis type 1 (*NF1*), SDH complex assembly factor 2 (*SDHAF2*), transmembrane protein 127 (*TMEM127*), and MYC-associated factor X (*MAX*). Other pertinent features of these mutations are the fact that *SDHB* mutations carry a risk for malignant transformation, while alterations in *RET, TMEM127,* and *SDH* genes are associated with multifocal tumors. Somatic gene mutations in *VHL, MAX, RET, NF1, SDH*, as well as *HIF2α* belies 17 % of sporadic PCCs and PGLs.

17.3.4 Molecular Pathology of MENs

Multiple endocrine neoplasias (MENs) are a conglomerate of tumors that arise from the unrelated neuroendocrine cells that secrete most of the nonsteroidal hormones of the body, primarily amine precursor uptake and decarboxylation (APUD) cells. These diseases are inherited in an autosomal dominant fashion. The multiple endocrine neoplasia syndromes involve genes important in the regulation of neuroendocrine functions, and hence abnormalities lead to multiple neuroendocrine tissue involvement. There are three types based on genetic abnormalities and primary tissues involved. These are MENI, MENIIA, and MENIIB.

17.3.4.1 MENI

MENI is a rare disease, with an incidence of 0.25 % and prevalence of 0.02 to 0.2 per thousand. Despite its rarity, penetrance is very high. This disease is characterized by hyperplasia or neoplasm of endocrine pancreas, parathyroid, and pituitary glands. These abnormalities lead to hyperparathyroidism in 90 % of the patients, pancreatic islet cell tumors, mostly islet cell gastrinoma, which occurs in ~60 % of the cases, and pituitary adenomas that are found in ~40 % of the patients. Molecularly, MENI is characterized by mutations, mostly nonsense mutation (~80 %) in the *MEN1* gene located on chromosome 11q13 that encodes a 610 amino acid protein called menin. While its functions are not fully elucidated, menin interacts with multiple transcriptional regulators that control cellular proliferation.

17.3.4.2 MENIIA/B

MENII is an autosomal dominant disease with high degree of penetrance. Based on the structures mostly involved, MENII is further divided into MENIIA and MENIIB. All MENIIA patients will have calcitonin-secreting parafollicular or C cell hyperplasia or medullary ThyCa (MTC); 50 % will present with pheochromocytoma and 20–30 % will demonstrate hyperparathyroidism. Similarly, almost all MENIIB patients will have MTC and pheochromocytomas but rarely have hyperparathyroidism. Additionally, MENIIB patients may demonstrate other gastrointestinal neuroendocrinopathies such as ganglioneuromatosis of the gastrointestinal tract. Molecular pathologically, mutations in the *RET* tyrosine kinase proto-oncogene, which is expressed by tissues of neural crest origin, are implicated in MENII syndromes. All MENIIA patients have *RET* mutations, but a few MENIIB patients lack these mutations. Additionally, LOH at chromosomes 1p, 3p, 17p, and 22q are associated with MENII.

17.4 Circulating ThyCa Biomarkers

ThyCa biomarkers of clinical relevance are detection of $BRAF^{V600E}$ mutations in ctDNA and measurement of the circulating levels of thyroglobulin (TG or Tg) transcripts and antibodies. New members under investigation include circulating miRNA, circulating ThyCa cells, and circulating endothelial cells.

17.4.1 Circulating Cell-Free Nucleic Acid as ThyCa Biomarkers

Data on ccfDNA content or levels for ThyCa detection are lacking; however, studies of the epigenome and genome alterations in ccfDNA suggest their potential in ThyCa management. Methylation and loss of function of genes involved with iodine metabolism (*SLC5A8, SLC26A4*) occur in ThyCa, and *SLC5A8* hypermethylation is associated with *BRAF*^{V600E} mutation, the most common genetic alteration in PTC. Because of these associations, the levels of ccfDNA as well as alterations in these genes were assayed in ccfDNA of ThyCas of all histologic types. CcfDNA levels highly discriminated cancer from healthy controls and correlated with various histologic subtypes of ThyCa. A panel consisting of ccfDNA, methylation of *SLC5A8* and *SLC26A4*, and *BRAF*^{V600E} mutations demonstrated some diagnostic potential [1].

Similar to melanoma, a majority of PTC harbors *BRAF*^{V600E} oncogene mutations. While this mutation was absent in benign adenomas, follicular neoplasms or carcinoma, and thyroid lymphoma, 35.7 % of PTCs were positive for this mutation of which three were detected in serum samples as well, and these were associated with lymph node metastasis [2]. In contrast to the above low frequency of detection, Kim et al. found the mutation frequency to be 68.1 % in PTC tissue samples, but only three patients with lymph node and lung metastasis had detectable circulating *BRAF*^{V600E} mutations [3]. Pupilli et al., however, measured the percentage of *BRAF*^{V600E} in ccfDNA, which was significantly different between patients and controls, as well as different histologic categories [4]. Patients with PTC histology had higher percentage of the mutation than those with benign histology. As a marker of tumor removal, the levels fell after surgery. Used as a diagnostic, circulating levels of *BRAF*^{V600E} achieved an AUROCC of 0.797 at a sensitivity of 80 %, specificity of 65 %, PPV of 33 %, and NPV of 80 %.

17.4.2 Circulating ThyCa Coding RNA Biomarkers

Primarily TG mRNA has been investigated for its utility in diagnosis and recurrence monitoring. Recurrences of ThyCa are monitored by serum TG immunoassays and ^{131}I whole-body scans. But antibodies can hinder the sensitivity of the immunoassay. Hence, RT-PCR-based transcript measurements have been explored for such clinical utility. Thyroid-specific transcripts targeting *TG, TPO, TSHR, NIS*, and *PDS* are known biomarkers of recurrence or presence of residual cancer after surgery. These transcripts in circulation were measured and compared to the performance of serum TG protein levels. Overall, serum TG protein assay was superior. TG levels were higher in serum samples from cancer patients and much higher in those with residual tissue or metastatic disease during thyroid hormone stimulation test (THST) or recombinant human thyrotropin (rhTSH) stimulation.

Patients with relapse had increased serum TG levels compared to disease-free patients, and THST and rhTSH stimulations had 81.4 % and 90.9 % accuracy, respectively. The transcripts had lower sensitivities and specificities, but a panel of *TPO* and *TSHR* transcripts may be specific for detection of relapse [5]. In another study, circulating *TSHR* mRNA measured in comparison to *TG* mRNA as well as serum TG protein levels revealed a sensitivity of 100 % and specificity at 98 % for *TSHR* mRNA in detecting differentiated ThyCa. Additionally, *TSHR* mRNA had 95 % correlation with *TG* mRNA and protein levels. The detection in preoperative patients indicates its possible utility in ThyCa detection in people with undiagnosed thyroid nodules [6]. Circulating *TSHR* mRNA for preoperative diagnosis of ThyCa in patients with thyroid nodules was compared to FNA cytology and to serum TG levels and/or whole-body ^{131}I scan. *TSHR* mRNA levels were significantly increased in cancer patients compared to controls. At an established cutoff, circulating *TSHR* mRNA correctly detected ThyCa at a sensitivity of 72 % and specificity of 82.5 %. *TSHR* mRNA and ultrasound features of follicular lesions correctly classified all follicular cancers and potentially could have saved 31 % of patients with benign lesions from unnecessary surgery. Postoperative day 1 levels of *TSRH*, as a measure of circulating cancer cells, were predictive of disease status. Elevated levels were indicative of residual or metastatic disease [7]. *TSHR* mRNA in circulation has also been explored as an adjunctive predictive biomarker to monitor ThyCa recurrence. *TSHR* mRNA was correlated with TG immunoassay, imaging analysis, and disease status. Overall, TSHR mRNA was better than TG and agreed with whole-body scan detection of recurrent disease in 79 % of cases. *TSHR* mRNA also predicted disease status in 77 % of patients. In conjunction with TG immunoassay, this panel achieved a sensitivity of 90 % and specificity of 94 % in detecting cancer recurrences. Note however that this group comprised mainly papillary-type ThyCa patients (91 %) [8].

TG mRNA in circulation was detected in only 21.8 % of patients and was not a predictive marker of recurrence after thyroidectomy. Note the small percentage of detection could have influenced the outcome [9]. Earlier report by this same group had indicated that *TG* mRNA appears useful for predicting recurrence in PTC patients [10]. In this study, only patients with PTC had detectable *TG* mRNA in circulation (26.4 %). *TG* mRNA could not be detected in any of the other histologic types. In this subgroup, the assay had a sensitivity of 100 % and a specificity of 75 % in predicting the results of ^{131}I whole-body scans. In a cohort of patients with thyroidectomy and ^{131}I scanning, there was a significant difference in the median *TG* mRNA levels between those with no detectable thyroid tissue (no uptake) and those with thyroid bed uptake ($p < 0.0009$) and those with detectable thyroid tissue elsewhere in the body ($p < 0.010$). This assay proved useful for predicting disease behavior after surgery. *TG* mRNA had greater sensitivity, but similar specificity to TG immunoassay, and is unaffected by anti-TG antibodies [11].

17.4.3 Circulating ThyCa Noncoding RNA Biomarkers

Several deregulated miRNAs have been uncovered in ThyCa tissue samples using PCR and microarray analyses. Upregulated and with possible oncomiric functions are miR-21, miR-31, miR-34a, miR-122a, miR-146, miR-155, miR-172, miR-181, miR-187, miR-205, miR-213, miR-220, miR-221/222, miR-223, and miR-224 in PTC; miR-146, miR-155, miR-183, miR-187, miR-197, miR221/222, miR-224, and miR-339 in FTC; let-7a-2, let-7f-1, miR-7, miR-10a, miR-16, miR-137, miR-155, miR-187, miR-192, miR-194, miR-196a, miR-198, miR-200b, miR-205, miR-214, miR-221/222, miR-224, miR-302c, and miR-429 in ATC; miR-9, miR-9*, miR10a, miR-21, miR-124a, miR-127, miR-129, miR-137, miR-154, miR-183, miR-224, miR-323, miR-370, and miR-375 in MTC. Similarly, downregulated miRNAs with possible tumor suppressormiric functions include miR-1, miR-19b-1,2, mir-20a, miR-26a-1, miR-30a-5p, miR-30c, miR-130b, miR-138, miR-145sh, miR-218, miR-219, miR-292, miR-300, miR-345, and miR-886-3p in PTC, and miR-26a, miR-30a-5p, miR-30d, miR-125b, and miR-138 in ATC.

The biologic functions of some of these deregulated miRNAs in ThyCa have been revealed. MiR-221/222, miR-146, and miR-17-92 clusters appear to drive cell cycle progression, cell growth, differentiation, survival, and invasion. MiR-221/222 and miR-17-92 clusters target *PTEN* to activate the PI3K pathway, while targeting of *CDKN1B*/*p27* and *CDKN1C*/*p57* by miR-221/222 and *CDKN1A*/*p21* by miR-17-92 drives cell cycle progression. Moreover, two inhibitors of MMPs, *TIMP3* and *RECK*, are targets of miR-17-92 (*TIMP3*) and miR-221/222 (*RECK*) used to promote invasion. The NF-κB signaling pathway that is engaged in aggressive PTC enhances miR-146 expression to mediate cancer cell migration and invasion partly through *SMAD4* targeting by miR-146. ThyCa tumor suppressormirs also contribute to carcinogenesis. Loss of let-7 enhances MAPK signaling and induces cell migration through loss of *FXYD5* repression. The decreased expression of miR-30 family de-represses *EZH2*, a component of PRC2 involved in epigenetic regulation of gene expression. Another target of miR-30 is the regulator of autophagy, *BECN1*. MiR-200 family target *ZEB1*, *ZEB2*, and *CTNNB1* involved in EMT. Thus loss of miR-200 promotes tumor metastasis. Telomerase activity is enhanced partly through loss of miR-138 because of loss of control of its target, *hTERT*.

17.4.3.1 Circulating ThyCa miRNA Biomarkers

Only a handful of studies have addressed the clinical relevance of circulating miRNA in ThyCa. Global and targeted screening of blood samples has uncovered a number of miRNAs with differential circulating levels in PTC patients. Using Solexa sequencing followed by qRT-PCR validation of sera from patients with PTC and benign thyroid nodules and healthy controls, elevated levels of lef-7e,

miR-151-5p, and miR-222 were demonstrated in PTC patients compared to the two control groups [12]. In addition to their diagnostic potential, these miRNAs were associated with nodal status, tumor size, multifocality, and TNM stage. Circulating levels of miR-151-5p and miR-222 decreased after surgery. Plasma levels of miR-146b and miR-222 were markedly elevated in patients with recurrent PTC compared to those without recurrent disease [13]. The preoperative levels of these two miRNAs were equally higher in ThyCa patients compared to healthy control subjects, and thyroidectomy was associated with significant reductions in their levels. This group further assessed the circulating levels of miR-146b, miR-155, miR-221, and miR-222 in patients with benign thyroid nodules and those with PTC with or without nodal involvement. The levels of miR-146b and miR-155 were higher in PTC patients compared to those with benign nodules, and both demonstrated modest diagnostic utility with AUROCC of 0.649 for miR-146b and 0.695 for miR-155. Nodal involvement was associated with increases in the levels of miR-146b, miR-221, and miR-222. Circulating miRNAs for use in differential diagnosis of thyroid nodules were sought using TaqMan Array Human MicroRNA Cards [14]. Pooled sera from PTC patients, those with nodular goiter, and healthy controls were used to uncover eight differentially expressed miRNAs between the PTC patients and the control groups. Of these, miR-29b, miR-95, and miR-579 (decreased levels) and miR-190 (increased levels) were validated in an independent cohort of samples. MiR-95 alone achieved a diagnostic sensitivity of 94.9 %, which was increased to 100 % when combined with miR-190 in a multivariate risk model.

17.4.4 Circulating ThyCa Protein Biomarkers

17.4.4.1 Circulating Tg-Abs as ThyCa Biomarker

Antibodies to Tg are in circulation of patients with ThyCa and have been explored for clinical applications. Thyroglobulin is a 660-kDa glycoprotein encoded by TG gene. The mRNA is an 8.7 kb transcript from a 300 kb genomic DNA. Due to alternative slicing, heterogeneous molecules of Tg are found in circulation. Tg is a precursor molecule for thyroid hormone synthesis, and hence normal and differentiated ThyCa (DTC) cells usually produce this. The amounts produced by poorly differentiated and ATC cells are considerably low.

Tg measurements have proven useful for the postoperative (surgery or radioactive iodide ablation) follow-up management of DTC patients. It is currently used, in conjunction with ultrasound, for patient care, obviating the possible need for whole-body scans. In view of the inherent issues with Tg measurements, a number of recommendations have been provided to help improve its clinical utility. Some considerations before the use of Tg measurements in postoperative management of DTC are:

- Given that only ~67 % of DTCs secrete Tg, the preoperative secretory state of the tumor should be determined. Normal preoperative levels make it difficult to interpret undetectable postoperative levels because the tumor may lack the ability to secrete Tg. Indeed, small tumors with elevated preoperative Tg levels make the interpretation of postoperative levels reassuring.
- Preoperative samples should be taken before FNA biopsy or two weeks after the procedure to ascertain accurate determination of Tg levels.
- Tg has a half-life of 2–4 days, and hence levels fall following surgery. The release of Tg as a result of surgery should fall within 2 months after surgery. Thus, Tg levels reflect remnant thyroid tissue and TSH levels. If thyroid hormone treatment is instituted following surgery, only thyroid tissue remaining after surgery or metastatic deposits elsewhere in the body determine circulating Tg levels. In near total thyroidectomy, with serum TSH maintained at <0.1 mU/L, Tg concentration of <2 ng/ml is expected.
- In patients with elevated preoperative Tg levels, disease recurrence after surgery is associated with increases in Tg levels (with stable TSH levels). Knowing the preoperative Tg levels, the extent of thyroid tissue removed at surgery, and serum TSH levels enables good interpretation of postoperative measurements of Tg.
- Thyroidologists categorize DTC patients into low-risk and high-risk groups, who are monitored differently using Tg measurements. In low-risk patients, Tg < 0.5 ng/ml on suppression with rhTSH stimulation and measurement of Tg (rather than scanning) is satisfactory. A TSH-stimulated Tg cutoff level of 0.1 ng/ml is safe for this group. Patients in the high-risk category have different management protocols that may need scanning following a rise in Tg levels.
- Some adjustments are made with the development of sensitive and reliable assays that accurately measure extremely low Tg levels. With the use of these assays, a basal or suppressed Tg > 0.5–1.0 ng/ml may be considered abnormal. Undetectable Tg levels are associated with complete remission and may not require Tg stimulation tests. For these assays, even detectable levels <0.5 ng/ml may need supplementation with stimulated Tg measurements.

17.4.4.2 Circulating ThyCa Proteomic Biomarkers

A number of proteomic approaches have been applied to serum samples from ThyCa patients to uncover features and identify protein biomarkers for the management of this disease. Indeed, accurate diagnosis of ThyCa can be a difficult process given that the gold standard (FNAB) can be inconclusive in ~30 % of cases. While these proteomic studies are primarily level 3 evidentiary studies, they demonstrate the potential in augmenting disease detection.

Villanueva et al. determined that older age and gender have no effect on serum peptidome profiles in ThyCa patients [15]. In this discovery study, a 12-peptide ion ThyCa signature could classify patients in an independent validation cohort at 95 % sensitivity and specificity. While ten of these peptides were shared with other solid tumors, two, including dehydro-Ala(3)-fibrinopeptide A, were unique to ThyCa and

thus deserve further study. In another study, this group developed a functional proteomic approach referred to as sequence-specific exopeptidase activity test (SSEAT) that compares defined exoprotease activity between groups [16]. This test was applied to sera from patients with metastatic ThyCa and matched healthy controls. Without prior feature selection, this test had a diagnostic sensitivity and specificity of 94 % and 90 %, respectively. Wang et al. uncovered preoperative early detection biomarkers using SELDI TOF MS applied to 116 sera [17]. Two biomarker patterns were uncovered. Pattern 1 could differentiate PTC patients from healthy people, while pattern 2 distinguished PTC patients from those with benign thyroid nodules. In a small independent sample set, pattern 1 achieved a sensitivity and specificity of 88.9 % and 80.0 %, respectively, and pattern 2 had a sensitivity and specificity of 80 % each. Two additional peptide patterns were clinically useful; pattern 3 differentiated different stages of PTCs at 77.1 % accuracy, while pattern 4 was associated with pathologic types of ThyCa at 88.1 % accuracy. A group from the MSKCC Protein Center developed an automated platform for capturing LMW peptide patterns in serum samples [18]. In a pilot study, serum samples from patients with metastatic thyroid cancer and controls were analyzed using this platform. Serum profiles of 98 discriminatory LMW peptides could accurately separate the two groups.

Linkov et al. used xMAP profiling of cytokines, chemokines, and growth factors in sera from ThyCa patients, patients with benign nodules, and healthy controls [19]. In univariate analysis, five factors could separate patients with thyroid disease from healthy subjects. In multivariate analysis, IL-8, HGF, monocyte-induced γ IFN, and IL-12 p40 could distinguish patients with benign nodules from malignant thyroid disease, achieving an AUROCC of 0.81. To uncover noninvasive diagnostic biomarkers for PTC, sera from 108 PTC patients and 116 healthy controls were randomly divided into training and test sets [20]. SELDI TOF MS peak discovery followed by HPLC purification, LC tandem MS protein identification, and ProteinChip immunoassay validation enabled the discovery of three discriminatory peaks/peptides at *m/z* 9190 (haptoglobin α-1 chain), 6631 (apolipoprotein C I), and 8697 (apolipoprotein C III). These biomarkers were used to construct a classification discriminatory model that was applied to a blind test set achieving a sensitivity of 95.15 % and a specificity of 93.97 %. Haptoglobin α-1 chain levels were high, while apolipoprotein C I and apolipoprotein C III levels were low in PTC patient samples. These levels progressively mirrored tumor stage from I to IV.

The loss of ^{131}I uptake by metastatic PTC is a major obstacle to radioiodine therapy. Protein biomarkers that will enable stratification and/or monitoring of "uptakers and non-uptakers" will therefore be clinically useful. To uncover serum biomarkers that distinguished mPTC patients with and without ^{131}I uptake in lung metastasis, sera from PTC patients with ^{131}I-avid lung metastasis and cases with non-^{131}I-avid lung metastasis were analyzed [21]. A subset of the samples were used for decision tree model development and the remainder as blind test set. Of 151 protein peaks detected between m/z 1300–15,000, seven were significantly different between the groups ($p < 0.05$). The blind validation test achieved a sensitivity of 92.6 % and specificity of 85.7 %. In another study by this group, MALDI TOF MS and validation by western blotting enabled identification of

afamin as a decreased serum biomarker in patients with mPTC with non-^{131}I-avid lung metastasis [22]. Afamin could be a biomarker for making radioiodine treatment decisions.

The secretome of aggressive and nonaggressive ThyCa cell lines was uncovered [23]. A panel of candidate biomarkers were then analyzed in patients' serum and tissue samples. Elevated presurgical activated leukocyte cell adhesion molecule (ALCAM /CD166) levels were associated with aggressive tumors ($p = 0.04$), and nodal involvement ($p = 0.018$). Similarly, elevated levels of tyrosine-protein kinase receptor (AXL) were associated with extra-thyroidal spread ($p = 0.027$).

17.4.5 Circulating ThyCa Cells

The clinical relevance of circulating ThyCa cells (CThyCaCs) has been addressed primarily by molecular approaches targeting thyroid-specific transcripts such as Tg, TSHR, calcitonin/CT, and TPO. Circulating Tg is traditionally used to manage ThyCa patients. However, there are issues such as anti-Tg antibodies that affect its measurements. Tg mRNA assays were thus developed to overcome these limitations. In DTC patients on thyroid hormone for TSH suppression, Tg mRNA was present in 79 % of the patients compared with 36 % positive for serum Tg. Of interest, circulating epithelial cells that stained strongly with anti-Tg antibodies were present in peripheral blood of these patients [11, 24]. Novosel et al. initially demonstrated the presence of TSHR-mRNA expressing circulating cells in 59 % of patients with papillary thyroid microcarcinoma (PTmC), and their presence potentially identified patients with aggressive disease [25]. Using TSHR mRNA as a marker of CThyCaCs, 46 % of patients with PTmC, and 80 % of patients with macroscopic PTC were positive. In older patients ($\geq$45) with PTmC, the presence of TSHR-positive CThyCaCs predicted the likelihood of lymph node metastasis [26].

Other markers used to characterize CThyCaCs include *KRT, preproGRP, CEA*, calcitonin (*CGRP*), thyroid peroxidase (*TPO*), and *RUNX2* mRNAs. To improve ThyCa detection and to assess disseminated tumor cells, *KRT20* mRNA was measured in blood from patients with cancer, benign thyroid diseases, and healthy individuals. Using this as a measure of CThyCaCs, 25.8 % of ThyCa patients were positive, while none of the control samples were [27]. In a follow-up study, *KRT20* and preprogastrin-releasing peptide (preproGRP) mRNAs were used to detect CThyCaCs in patients with MTC. All tissue samples expressed *KRT20* and *preproGRP*, while 67 % and 72 % of lymph nodes were positive for *KRT20* and *preproGRP*, respectively. In peripheral blood, each biomarker was positive in 28 % of the patients, and *preproGRP* positivity was significantly associated with tumor stage [28]. *KRT20* mRNA detection in blood correlates with tumor categories, being positive in 71 % of FTC, 47 % of PTC, and 14 % of ATC tissue samples. In 41 % of *KRT20*-positive carcinomas, disseminated tumor cells were detected, and this was associated with local and distant metastasis and hence poor prognosis [29].

Established MTC markers are serum calcitonin and CEA levels. Thus, *CGRP* and *CEA* mRNA as markers of CThyCaCs were used as biomarkers of early disease recurrence. While *CGRP* mRNA was undetectable in circulation, *CEA* mRNA was positive in 72.7 % of MTC patients compared with 34.4 % of normal controls [30]. In the setting of MTC, *CEA* mRNA measurement may help detection of patients with relapse. *CEA* mRNA detection is associated with advance ThyCa. It was undetectable in benign and noninvasive cancer patients, but present in 46.2 % of patients with invasive FTC [31]. In blood of MTC patients with elevated serum calcitonin, CThyCaC detection targeting *CGRP* mRNA was present in 31.6% of patients (the low detection rate may be because many patients had total thyroidectomy) [32]. CThyCaC detection was closely associated with patients who had distant metastasis (62.5 % of patients were positive) and locoregional spread. CThyCaC detection by *CGRP* mRNA appears specific to cancer patients, as they were undetectable in healthy controls and patients with benign thyroid diseases. As a possible early detection of micrometastasis, *TPO* and *TSHR* mRNAs were examined in blood as markers of CThyCaC [33]. *TPO* mRNA was undetectable in healthy controls, and present in only 4 % of patients with benign thyroid diseases, but was in as many as 61 % of patients with early stage I PTC. This evidence shows potential as early detection biomarker of micrometastatic disease. *RUNX2* mRNA was significantly much higher in serum and circulating non-hematopoietic cells from PTC patients than samples from healthy controls [34]. Cancer patients with microcalcifications also demonstrated significantly higher serum *RUNX2* mRNA than those without.

17.4.6 Circulating Epithelial Cells as ThyCa Biomarkers

Studies primarily from a single center demonstrate the clinical relevance of circulating epithelial cells (CECs) in ThyCa patients. The clinical relevance of EpCAM expressing CECs has been examined primarily in patients with DTCs [35]. In this cohort, CECs were detected in DTC and athyreotic patients as well as normal controls. However, the number of CECs was much higher in patients who received surgical thyroid removal than those without surgery (surgery-induced release of CECs). Also in patients with active DTC, the numbers of CECs were higher than the control group, and the number of CECs correlated with Tg levels. In DTC patients on radioiodine therapy (RIT), early decreases in CECs were associated with increases in Tg levels (due to cell death from therapy) [36]. In a subset of patients with metastatic disease on repeated RIT, a decrease in CECs 2 days after therapy indicated clinical response in 90 % of the patients. CECs as a measure of disseminated tumor cells in DTC patients were assessed targeting *Tg, TSHR, TPO*, and *NIS* mRNAs at a single cell level [37]. At least three markers were positive in 33.3 % of cells, and this was associated with patients who had detectable serum Tg.

17.5 Adrenal Cortical Carcinoma Biomarkers in Circulation

Very limited studies have addressed the role of circulating biomarkers in ACC patients. MiRNAs and CTCs may have a role in the diagnosis and management of patients with ACCs. In 2013, Chabre et al. performed a prognostic prediction study using tumor tissue and serum samples from patients and controls [38]. Of eight miRNAs selected for validation following microarray screening, five were assayed in sera from healthy controls, patients with adenomas, and non-aggressive and aggressive ACCs. Circulating miR-195 and miR-335 were significantly decreased in both tumor and serum samples from ACC patients compared to noncancerous controls. In tissue samples, miR-139-5p and miR-376a were significantly elevated in aggressive compared to non-aggressive cancers. Importantly though, the upregulated tissue miR-483-5p was detected in sera from only patients with aggressive ACC. High serum miR-485-5p was significantly associated with prognostic variables of shorter recurrence-free ($p = 0.0004$) and overall ($p = 0.0005$) survival. Similarly, low levels of circulating miR-195 were significantly related to both shorter RFS ($p = 0.0014$) and OS ($p = 0.0086$). Szabo et al. addressed the diagnostic role of circulating miRNAs in patients with ACC [39]. In ACC and ACA patients, five miRNAs (miR-100, miR181b, miR-184, miR-210, and miR-483-5p) that were deregulated in ACC tissue samples were found elevated in plasma samples from ACC patients. The diagnostic AUROCC revealed the best plasma diagnostic miRNAs were a combination of miR-210 and miR-181b (AUROCC of 0.87) as well as the ratio of miR-100 to miR-181b (AUROCC of 0.85).

Pinzani et al. addressed the role of CTC characterization as a potential biomarker for ACC management [40]. CTCs were isolated by size filtration using ScreenCell devices and characterized by immunohistochemistry targeting markers of adrenal cortex such as MART1, SF1, and synaptophysin. Of interest, CTCs were detected in all blood samples from ACC patients ($n = 14$) but not in any of the samples from ACA patients ($n = 10$). The number of CTCs in postsurgical blood samples correlated with tumor stage and diameter. This pilot study has implications for CTC characterization in the management of ACC patients.

17.6 PCC and PGL Biomarkers in Circulation

17.6.1 Circulating Catecholamines as PCC and PGL Biomarkers

In order to appreciate the routine biochemical assays used to screen for PCC and PGL, a basic knowledge of catecholamine biosynthesis and metabolism is essential. Catecholamine biosynthesis begins with tyrosine hydrolase-mediated conversion of

tyrosine to 3,4-dihydroxyphenylalanine (DOPA), which is further converted to dopamine, and subsequently noradrenaline (norepinephrine) by dopamine β-hydroxylase. In the adrenal medulla, phenylethanolamine-N-methyltransferase (PNMT) converts noradrenaline to adrenaline. Because adrenal medullary cells almost exclusively express this enzyme, almost all adrenaline in the body is from this tissue. Catecholamine is degraded into various metabolic intermediates and end products. However, catechol-O-methyltransferase (COMT) in adrenal medullary chromaffin cells metabolizes adrenaline and noradrenaline to produce metanephrine and normetanephrine, respectively. Dopamine is also converted to methoxytyramine. All these molecules serve as circulating biomarkers of PCC/PGL.

The standard diagnostic work-up for suspected patients includes measurement of circulating and 24-h urinary catecholamines, methoxytyramine, and granins. The catecholamines, adrenaline and noradrenaline, as well as their metabolites, metanephrine and normetanephrine, are diagnostic biomarkers of PCC and PGLs. Because some tumors (especially those in asymptomatic patients) do not secrete catecholamines and that even secreting tumors do so episodically, it is advocated that plasma and/or urinary metanephrine measurements be the first-line biochemical test of choice. This decision is due to the demonstration that metanephrine release from tumors is a continuous process compared with the episodic secretion of other catecholamines. The diagnostic sensitivity and specificity of free plasma metanephrine range between 96–99 % and 80–100 %, respectively. Unlike circulating metanephrines, normetanephrine levels are influenced by age such that the upper cutoff limit is 0.62 nmol/l for individuals <40 years of age and 1.05 nmol/l for people >60. The established upper limit of normal for metanephrine is 0.45 nmol/l. However, an almost perfect diagnostic specificity is obtained with elevated free plasma metanephrine and normetanephrine levels above 1.2 nmol/l and 2.2 nmol/l, respectively.

17.6.1.1 Association of Circulating Catecholamines with Tumor Features

Evidence suggests circulating catecholamines and their metabolites may predict the underlying mutations in PCC and PGLs. Work by Eisenhofer et al. suggests that elevated levels of metanephrine are associated with *NF1* and *RET* mutations [41]. Likewise, lack of adrenaline and increased circulating levels of normetanepthrine is a feature of *VHL* and *SDH* mutation tumors. Thus, the measurement of these two biomarkers enabled the separation of these two-mutation clusters in 99 % of their series. Because tumors with *SDHB*/*D* mutations also demonstrate solitary secretions of 3-methoxytyramine, measurement of the three metabolites enabled 100 % separation of *VHL*/*SDH* from *NF1*/*RET* patients. Indeed, in 78 % of the cases, 3-methoxytyramine levels could further stratify *VHL* and *SDHB*/*D* patients. Finally, substantial increases in normetanephrine levels are associated with patients who have *MAX* mutations [42].

Because almost exclusively adrenal medullary chromaffin cells produce adrenaline (because PNMT is only expressed by these cells), noradrenergic tumors, primarily of extra-adrenal origin, secrete more norepinephrine and normetanephrine than adrenergic tumors. Expectedly, elevated circulating levels of adrenaline and metanephrine are features of tumors from the adrenal medulla. Because metastatic disease is frequently associated with extra-adrenal tumors (often with *SDHB* mutations), the levels of noradrenaline/normetanephrine can be used to predict tumor size and possible spread. Moreover, high circulating 3-methoxytyramine levels are associated with malignancy.

17.6.2 Circulating Granins as PCC Biomarkers

The granins are a family of glycoproteins referred to as chromogranins (Cgs) and secretogranins (Sgs). Neuroendocrine cells and tumors store these molecules together with their peptide and/or amine hormones in membrane-enclosed dense vesicles. They may constitute a substantial part of these secretory granules, making up to 80 % of the core proteins in some vesicles. Granins are involved in generation and stabilization of secretory granules and mediate protein trafficking. Their cleavage products perform some of these functions. They are co-released with the peptide hormones, and hence their levels are much elevated in patients with neuroendocrine tumors such as PCC, carcinoid tumors, SCLC, neuroblastomas, MTC, MENs, and ovarian and pancreatic endocrine tumors.

The chromogranins are composed of chromogranin A (CgA), chromogranin B (CgB), and their cleavage products. After establishing their elevated expression by chromaffin cells and tumors, O'Connor and Bernstein first demonstrated their elevated circulating levels in patients with pheochromocytomas (1614 $\pm$ 408 ng/l) compared to healthy controls (129 $\pm$ 12 ng/l) [43]. Following this initial report, several studies have confirmed and established the elevated circulating levels of CgA in patients with PCC. It has also been recognized that levels of CgA are much higher in PCC tissue samples from MEN2 than those from VHL patients. Hsiao et al. first demonstrated the diagnostic potential of circulating CgA in PCC patients [44]. In their cohort of patients, a sensitivity and specificity of 83 % and 96 %, respectively, were achieved. Since then, several investigators have confirmed the high performance of CgA in detection of PCC. Thus, numerous assays have been developed with different cutoffs for the measurement of circulating CgA. Specificity is compromised by confounding factors such as patients with renal impairment, heart failure, on proton pump inhibitors, and with type A gastritis. These must be considered in interpretation of assay results. The potential plasma biomarkers of Cgs include CgA-derived peptides such as pancreastatin, chromacin, WE14, and EL35. Guillemot et al. demonstrated a 5.4-fold increase in WE14 in PCC patients compared to healthy controls [45]. While the levels of this peptide improved the performance of EM66 and CgA assays independently, the combination of the three biomarkers achieved a high sensitivity of 95 % in PCC detection. Also elevated in

circulation of PCC patients are CgB (initially referred to as secretogranin I) and its derived peptide GAWK.

The secretogranins are composed of at least six members. They are identical to the Cgs in structure except the absence of the N-terminal hydrophobic disulfide-bonded loop found in Cgs. They are designated as SgII to SgVII. The circulating levels of SgII-derived peptides, secretoneurin and EM66, are elevated in PCC patients compared with healthy controls. Indeed, Guillemot et al. demonstrated that plasma levels of EM66 were tenfold higher in PCC patients than in normal volunteers and returned to normal following tumor removal [46]. Of the other Sgs, only SgV has shown increased plasma levels in patients with PCC (221 ± 82.8 ng/l) compared with healthy volunteers (55.8 ± 1.2 ng/l).

17.7 Summary

- The endocrine system is an array of tissues united by their shared functions in maintaining body homeostasis through secretions into the vasculature that exert their functions at distant sites.
- The most common tumors of the endocrine system involve the thyroid gland.
- Thyroid tumors are mostly benign, but a few (~5 %) are malignant. Mortality from ThyCa is generally low, but deaths in the resource-poor parts of the world almost double those in the developed world.
- Deregulated MAPK, WNT/β-catenin, PI3K, and cell cycle pathways underlie thyroid carcinogenesis.
- Circulating tumor DNA as detected by epigenetic (e.g., *SLC5A8* methylation) and genetic (e.g., $BRAF^{V600E}$) alterations are clinically relevant noninvasive ThyCa biomarkers.
- Other noninvasive biomarkers of ThyCa are circulating transcripts (TG mRNA) and TG antibodies.
- Circulating miRNA are emerging noninvasive ThyCa biomarkers, while proteomic efforts are ushering in novel proteins.
- The role of circulating ThyCa and endothelial cells as ThyCa biomarkers remains to be clarified.
- There is paucity of circulating biomarkers for adrenal cortical tumors.
- Circulating biochemical markers of PCC and PGL are catecholamine and their metabolites, as well as chromogranin A, B, and their cleavage products.

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Chapter 18
Brain Tumor Biomarkers in Circulation

Key Topics

- Molecular pathology of brain tumors.
- Circulating glioblastoma (GBM) epigenetic biomarkers.
- Circulating GBM miRNA biomarkers.
- Serum GBM protein biomarkers.
- Circulating GBM cells.
- Circulating endothelial and endothelial progenitor cells.

Key Points

- GBM, the most common brain cancer is associated with dismal median survival of 12–18 month due to the spread of cancer cells to other parts of the brain and hence are not easily amenable to complete surgical removal.
- The molecular pathology of GBM involves epigenetic and genetic changes that alter the RTK/RAS/MAPK, PI3K, cell cycle, and growth factor signaling pathways.
- The molecular pathologic changes, especially *MGMT* methylation, *IDH1*/*IDH2*, and *EGFRvIII* mutations, are detectable in circulation as adjuncts to disease management. Emerging circulating GBM biomarkers include miRNAs, serum proteins, circulating GBM cells, endothelial cells, and extracellular vesicles.

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18.1 Introduction

Nervous system (NS) tumors are a conglomerate of neoplastic transformation of cells that constitute the nervous system. While there are numerous types, gliomas are the commonest. Gliomas constitute a group of central NS (CNS) tumors further subdivided based on putative cell of origin into astrocytomas, oligodendrogliomas, and oligoastrocytomas. Globally, ~200,000 people are afflicted annually with a diagnosis of glioma. While not as common as other solid tumors such as colon cancer, the prognosis of glioma is very dismal. In the US, 23,770 new cases of nervous system tumors and 16,050 deaths are expected for 2016. The recurrent rate is high, usually with the emergence of high-grade tumors. The median survival is 12–18 months for WHO grade IV glioblastoma (GBM). Even supposedly early stage (grade II) patients have just 5–10 years, while grade III oligodendroglioma and astrocytoma patients survive for 3–10 years and 2 years, respectively. The need for early detection and importantly predictive biomarkers for this tumor is thus urgently needed.

For personalized medicine, accurate classification of CNS tumors is needed. Thus, biomarkers that inform tumor behavior or biology ushers in this paradigm shift in management of brain tumors. As is established, co-deletion of chromosomes 1p and 19q in grade III anaplastic oligodendrogliomas is associated with a better prognosis than other glioma subtypes. Similarly, isocitrate dehydrogenase 1 (NADP+) *IDH1* mutations in grade III glioblastoma are associated with better prognosis than patients with *IDH1* wild-type tumors. There are currently emerging biomarkers, such as noncoding RNAs, proteomic, and metabolomic biomarkers, which should enhance our knowledge and management of gliomas. Being able to assay these biomarkers noninvasively and longitudinally will definitely improve glioma early detection and optimal patient care.

18.2 Screening Recommendations for Brain Tumors

There are no recommended screening guidelines for brain cancer for a number of reasons. First, because of the low incidence, the harms from screening outweigh any benefit. Second, the risk factors for the general population are obscure, such that no defined high-risk population is currently available to be targeted for screening. The only exceptions are individuals with heritable familial mutations such as neurofibromatosis types 1 and 2, tuberous sclerosis, VHL disease, and Li–Fraumeni syndromes. These families have elevated risk for many cancers including brain cancer and hence require close surveillance. Similarly, people exposed to toxic radiations or those with impaired immune systems such as AIDS patients need close monitoring. While noninvasive tests are being developed for this high-risk group, current screening involves a variety of imaging modalities, including functional MRI and PET scans.

18.3 Molecular Pathology of Brain Tumors

The most common adult primary brain tumor with an estimated global incidence of 3.5/100,000 and one of the deadliest cancers is GBM. It is very lethal because, first, tumor cells infiltrate surrounding brain tissue making complete surgical removal impossible, and second, these tumors are often resistant to chemotherapy and radiation. Thus, the mean survival is 12–18 months.

Gliomas are classified based on cellular origins as determined by histology and immunohistochemistry, and graded (I–IV) based on aggressive features including mitotic activity, microvascular proliferation, and necrosis. GBM, the most aggressive form of gliomas (grade IV) originates via two mechanisms. Primary or de novo GBM, which is the more common type, develops quite rapidly usually following a short clinical history. GBM can also develop from low-grade gliomas such as diffuse astrocytomas (WHO grade II) or anaplastic astrocytomas (WHO grade III), and these are referred to as secondary GBMs. As expected, the two are molecularly distinct as well. Primary GBMs are characterized by *PTEN* and *EGFR* alterations, while *TP53* loss of function is more common in secondary GBMs.

18.3.1 Epigenetic Alterations in Brain Tumors

Gene promoter hypermethylation is a recognized feature of GBM. Of interest, methylation of *MGMT* is an established predictive biomarker in GBM. *MGMT* methylation, which occurs in ~45 % of adult GBMs, results in loss of gene function and hence reduced efficiency of DNA repair. The fragile state of such tumors is exploited for prediction of patients who will respond to temozolomide (TMZ). Thus, adult GBM patients with *MGMT* methylation benefit from TMZ chemotherapy, while those without do not. Similarly, the effect of this therapy in childhood high-grade glioma (HGG) is yet to be resolved. Methylation also affects a number of genes such as *TP53, RB1, CDKN2A, PTEN, PDGFβ*, epithelial membrane protein 3 (*EMP3*), protocadherin γ subfamily A, member II (*PCDHGAII*), and suppressor of cytokine signaling 1 (*SOCS1*) that have established roles in glioblastomagenesis.

18.3.2 Chromosomal Alterations in Brain Tumors

Glioblastoma is characterized by a wide spectrum of gene copy number alterations, as well as LOH. Deletions of *CDKN2A* and *CDKN2B* occur in ~53 % of adult GBMs and as many as 20 % of pediatric HGGs. *EGFR* amplification occurs in ~43 % of adult GBMs and <5 % of pediatric HGGs. These high frequency copy

number changes often occur concurrently with *EGFR* deletions and/or point mutations. In adult GBM, *high-level amplifications* are found in *CDK4* (~13 %), *PDGFRA* (~11 %), *MDM2* (~8 %), *MDM4* (~7 %), *MET* (~2 %), and *CDK6* (~1.5 %); *amplifications* occur in the following genes: *MYCN, MYC, CCND2, CCNE1*, and *SOX2*. Additionally, homozygous deletion of *RB1, CDKN2C, PTEN, FAF1, NF1, QKI, TP53*, and *NPAS3* characterizes adult GBM. Amplifications of *PDGFRA, MYC*, and *MYCN* are often more associated with pediatric cases.

Glioblastoma is a genetic disease with well-characterized genomic alterations. Multiple chromosomal structural abnormalities underlie GBM pathogenesis. The majority (83–85 %) of adult tumors harbor gains at chromosome 7 and loses at chromosome 10. These two alterations often occur concurrently in the same tumors and are therefore referred to as $7^{+}/10^{-}$ tumors. They are more common in tumors from people ≥70 than those ≤40. Other frequent chromosomal changes in adult GBM are gains at chromosomes 19 and 20 (occurs in 35–40 % of cases) and losses at chromosomes 9p (~38 %), 13q (~33 %), 22q (~33 %), 14q (27 %), and 6q (22 %). In general, pediatric HGG harbors less frequent chromosomal aberrations, except gains at chromosome 1q that occurs at a frequency of ~20 %.

In addition to the chromosomal gains and losses, a vast majority of GBM (~69 %) displays intergenic, intragenic, and interchromosomal rearrangements, and these alterations are commonly associated with copy number changes at the breakpoints. A classic example is the intragenic deletion of exons 2–7 in *EGFR* that encodes for the extracellular domain of the receptor (*EGFRvIII*), and these aberrations often coexist with amplifications and increased expression of the wild-type allele. Similarly, the increased expression of *PDGFR* is accounted for by amplifications and age-dependent intragenic deletion rearrangements. A few cases of GBMs also display gene fusions. *EGFR* activation can also occur as a result of recurrent translocation and fusion to *SEPT14* or *PSPH*, and interestingly, this often occurs at amplified regions of the fusion partner. Constitutive kinase activity of *FGFR1* in GBM may be caused by inversion and in-frame fusion to *TACC1* (similarly *FGFR3* can fuse to *TACC3* as in non-muscle invasive bladder cancer).

18.3.3 Mutations in Brain Tumors

The Cancer Genome Atlas (TCGA) data indicate that ~46 % of GBM harbors at least one somatic mutation that affects genes associated with DNA methylation (*IDH1*), histone modifications (*SETD2, HDAC, KDM6A, MLL2–4,* and *EZH2*), or chromatin remodeling (*ATRX, CREBBP, CHD5–9,* and SWI/SNF-related matrix-associated actin-dependent regulator of chromatin A2—*SMARCA2*).

The majority (58–84 %) of adult GBMs harbors mutational hotspots in *TERT* promoter that results in increased telomerase expression and activity. These mutations tend to occur in association with amplifications in *EGFR* and negatively correlate with mutations in *IDH1, IDH2, ATRX*, and *TP53*. Pediatric tumors tend

to harbor *ATRX* loss, *PDGFRA* mutations, and amplifications, and few (3–11 %) also have *TERT* mutations.

Other genes frequently mutated in adult GBMs are in order of decreasing frequencies: *PTEN* (~29 %), *TP53* (~29 %), *EGFR* (~20 %), *NF1* (~9 %), *SPTA1* (~9 %), *RB1* (~8 %), *PIK3CA* (~7 %), *ATRX* (~6 %), *PIKBR1* (~6 %), *IDH1* (~5 %), *KEL* (~5 %), *GABRA6* (~4 %), *LZTR1* (~3 %), *BCOR* (~2 %), *BRAF* (~2 %), *CTNND2* (~2 %), and *QK1* (~2 %).

Almost all the genetic lesions in GBM impinge on three main signaling pathways to promote tumor growth and spread. The RTK/RAS/PI3K pathway is activated in ~88 %, p53 pathway in ~87 %, and the RB pathway in as many as 78 % of GBMs.

18.3.4 Molecular Subtypes of GBM

Various subtypes of GBM have been identified based on methylation, gene expression, and genomic aberration data sets. Currently, at least six subtypes are recognized.

First, using unsupervised hierarchical clustering of TCGA expression data set, copy number alterations, and sequencing, Verhaak et al. subclassified GBMs into four molecularly distinct subclasses [1].

- Proneural subtype is enriched for *PDGFRA, CDK4, CDK6,* and *MET* aberrations and frequent *IDH1* mutations.
- Classical subtype harbors *EGFR* amplifications, as well as *PTEN* and *CDKN2A* loss.
- Mesenchymal subtype features mutations or losses in *NF1, TP53,* and *CDKN2A*.
- Neural subtype has no unique genetic signature.

Second, Noushmehr et al. similarly used unsupervised hierarchical clustering of DNA methylation profiles from the TCGA samples to delineate subtypes of GBM based on glioma CpG island methylator phenotype (G-CIMP) status [2]. This G-CIMP-positive subclasses are enriched in the proneural subclass of Verkhaak et al., and thus associated with *IDH1* mutations that could account for the G-CIMP.

Sturm et al. observed mutations in *H3F3A* and its chromatin remodeling complexes *ATRX* and *DAXX* in 44 % of pediatric cases [3]. Thus, they decided to include these in subclassification. Using epigenetic, genetic, and transcriptomics data from 210 GBM patient samples composed of cases from adults and children, six subtypes of GBM were mapped:

- Subtype 1 occurs mostly in young adults and is characterized by G-CIMP-positive, proneural gene expression, and *IDH1* and *TP53* mutations.
- Subtype 2 is a feature of childhood midline and brainstem GBMs, also expresses proneural genes, but has *H3F3A* (K27) mutation, and strongly positive for *OLIG2* staining. The prognosis of this subtype is very poor.

- Subtype 3 occurs mostly in teenagers and young adults and is characterized by G-CIMP-positive, *TP53*, and *H3F3A* (G34) mutations.
- Subtype 4 is similar to adult classical subtype with similar transcription map, *EGFR* amplification, and *CDKN2A* loss.
- Subtype 5 has profile of adult mesenchymal subtype but with relatively few mutations and copy number changes.
- Subtype 6 has adult proneural expression profile, but *PDGFR2A* amplification, and *CDKN2A* loss.

18.4 Circulating Brain Tumor Biomarkers

Circulating biomarkers complement GBM management especially with the development and deployment of targeted therapies. Thus, the molecular pathologic alterations including *MGMT* methylation, *IDH1*/*IDH2* mutations, and *EGFRvIII* are detectable in circulation. MiRNAs, serum proteins, circulating GBM, and endothelial cells (CECs), as well as extracellular vesicles, are all potential noninvasive GBM biomarkers (Fig. 18.1).

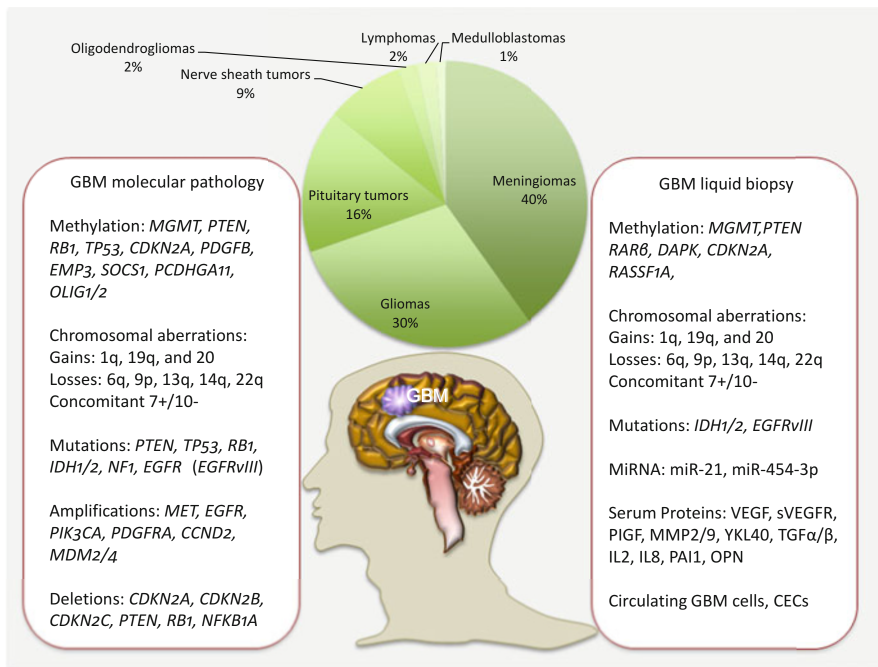

Fig. 18.1 Frequencies of brain tumors, GBM molecular pathology, and some liquid biopsy biomarkers

18.4.1 Circulating Brain Tumor Epigenetic Biomarkers

The epigenome has been studied in circulation of glioma patients. Using bisulfite sequencing, hypomethylation of ALU repeats in glioma tissues and matched serum samples from patients revealed high correlation ($r = 0.882$) between the two sample sets [4]. As a diagnostic biomarker, serum ALU hypomethylation achieved an AUROCC of 0.861 in glioma detection. Also, in tissue and serum samples, high methylation of ALU sequences correlated with longer survival. Methylation of multiple genes including *CDKN2A*/*P16*, *MGMT*, *RARβ*, and *TP73* in glioma tissues and plasma from patients was studied for the first time as a proof of principle [5]. Methylation in at least one gene was present in 90 % of the tumor samples, of which 67 % plasma samples harbored identical alteration in this small sample set. Another multi-marker methylation analysis targeted *MGMT*, *RASSF1A*, *CDKN2A*/*p16*, *DAPK,* and *TMS-1* in tumor tissue and matched serum samples from patients with GBMs [6]. The frequencies of methylation in tissue samples were *MGMT* (38.1 %), *CDKN2A*/*p16* (66.7 %), *DAPK* (52.4 %), and *RASSF1A* (57.1 %). Corresponding gene methylation frequencies in serum samples were 39.3 %, 53.6 %, 34.3 %, and 50 %. There was strong correlation between methylation in tissue and serum samples. *CDKN2A*/*p16* methylation in diffuse gliomas (astrocytomas and oligodendrogliomas) from the brain and brainstem as well as matched serum samples from patients and controls revealed methylation in 60 % of astrocytomas but in only one oligodendroglioma [7]. Similar methylations were detected in 75 % of serum samples from patients with astrocytomas.

A predictive study of response to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and temozolomide treatment of patients with GBM targeted methylation of multiple genes [8]. Both tumor and serum samples were assayed for methylation in *MGMT, CDKN2A*/*p16, DAPK*, and *RASSF1A*. *MGMT* methylation and silencing enhanced the response to BCNU treatment. Methylation in all the genes assayed correlated with serum methylation patterns. But only *MGMT* methylation predicted response to BCNU treatment. Overall survival and stability of disease were observed in 90.9 % of patients with *MGMT* methylation compared to only 35.7 % of patients without *MGMT* methylation. Similarly, time to progression was 29.9 weeks for patients with *MGMT* methylation compared to 15.7 weeks for patients without methylation.

18.4.2 Circulating Brain Tumor Genetic Biomarkers

MYC-N gene amplification in neuroblastoma is detectable in plasma and serum samples from patients, and may be useful in treatment monitoring. Tumor-specific epigenetic and genetic markers were assayed in serum from patients with gliomas (astrocytomas and oligodendromas) [9]. LOH on chromosomes 1p, 19q, and 10q, as

well as promoter methylation of *MGMT* and *PTEN* were assessed in serum samples. While the specificities were high, the sensitivities were only 51 % for LOH and 55 % for methylation targets in serum. Tumor-serum concordance was statistically significant for *MGMT* methylation (for both types of gliomas) and LOH at 1p and 10q (for oligodendromas).

18.4.3 Clinical Utility of Liquid Biopsy in GBM

The molecular pathology of glioma has provided lead biomarkers for disease management. The detection or measurement of *MGMT* methylation, 1p/19q co-deletion, *IDH1* and *2* mutations, *EGFR* amplification, and *EGFRvIII* expression offers clinical insights into various diseases. They serve as biomarkers for diagnosis, prognosis, treatment prediction, and monitoring. For example, 1p/19q deletion serves as a diagnostic, prognostic, and predictive biomarker of diffuse glioma. Additionally, these molecular alterations serve as targets for drug development. As examples, bevacizumab targets VEGF, the inhibitor AGI-5198 targets IDH1/2, and rindopepimut targets EGFRvIII. While traditionally tissue biopsies are used for these genetic analyses, liquid biopsy is paving the way for the noninvasive and longitudinal assessment of these molecular alterations in ctDNA. As demonstrated examples, promoter methylation of *MGMT* is both of prognostic and predictive relevance, *EGFRvIII* alterations are present in extracellular vesicles, and mutations in *IDH1*/*IDH2* are detectable in ctDNA.

18.4.4 Circulating GBM Noncoding RNA Biomarkers

MiRNA alterations in circulation of GBM patients have been addressed with some promise. Plasma miR-21 levels are altered in a number of neoplastic conditions. In GBM, Ilhan-Mutlu et al. observed a four-fold increase in a particular patient samples years before the clinical diagnosis of GBM (control group had significantly decreased circulating miR-21) [10]. In another study, miR-21 levels were significantly elevated in GBM patients ($p = 0.02$), and the levels dropped significantly ($p = 0.05$) after surgery. Increasing miR-21 levels were associated with a patient who developed recurrent disease [11, 12]. Li et al. identified miR-21 in exosomes derived from a GBM cell line (U251) [13]. Indeed, over 28 miRNAs were enriched in these exosomes, with higher levels of nine miRNA*s including miR-93*, miR-106a*, miR-181a*, and miR-452* than their matured counterparts. However, serum miR-21 has also been shown to be a diagnostic and prognostic biomarker of primary CNS lymphoma (PCNSL) [14]. The levels were significantly increased in PCNSL compared to other brain tumors and healthy controls, and this achieved a diagnostic accuracy with AUROCC of 0.930 and 0.916 in the test and validation data sets. Indeed, miR-21 could separate patients with PCNSL from GBM with an

AUROCC of 0.883 in the test set and 0.851 in the validation cohort. MiR-21 emerged in both Kaplan–Meier and multivariate Cox regression analysis as a strong independent predictor of OS of PCNSL.

MiR-21, miR-128, and miR-342-3p show differential plasma levels between GBM patients and healthy controls [15]. However, these miRNAs were not specific to GBM as they were associated with other brain tumors (meningiomas, pituitary adenomas) as well. Their levels correlated with histopathologic grades of glioma and returned to normal levels following surgery. This group subsequently uncovered miR-454-3p to be significantly elevated in plasma samples from glioma patients compared to healthy controls [16]. MiR-454-3p levels were higher in patients with high-grade than low-grade tumors. The diagnostic performance AUROCC was 0.9063. The levels decreased significantly following surgery, and high plasma miR-454-3p was significantly associated with poor prognosis. Serum miR-125b levels were significantly lower in glioma patients than healthy controls, and the levels correlated with WHO tumor grade [17]. As a diagnostic biomarker of glioma, serum miR-125b had an AUROCC of 0.839. This diagnostic accuracy improved with increasing tumor grade (grade II AUROCC was 0.868 and grade III–IV was 0.959 compared to grade I with an AUROCC of 0.691). But circulating miR-125b is not specific to glioma, as it performed equally well in detection of other cancers. Similar to miR-125b, circulating levels of miR-29 family were more useful at detecting advanced stage gliomas (AUROCC of 0.81) compared to early stage disease (AUROCC of 0.66) [18]. Again, this miRNA family is associated with other cancers as well, achieving an AUROCC of 0.83 in meta-analysis of other cancers.

Yang et al. used genome-wide Solexa sequencing to analyze sera from 122 untreated astrocytoma patients and 123 normal controls to uncovered seven altered miRNAs (miR-15b*, miR-23, miR-133a, miR-150*, mi-197, miR-497, and miR-548b-5p) that were validated by PCR as potential glioma signature biomarkers [19]. These were significantly decreased in grades II–IV astrocytomas ($p < 0.001$). As a panel, the seven miRNAs had a sensitivity of 88 % and a specificity of 97.87 % in detecting astrocytomas. Tissue samples also demonstrated decreased levels, and following surgery, serum levels increased significantly ($p < 0.001$).

18.4.5 Circulating GBM Protein Biomarkers

Global protein profiling of serum and plasma samples has been applied for the discovery of novel glioma biomarkers. Aside from proteomic approaches, there are other platforms with focused selected pathways or proteins of defined functions (e.g., cytokines, growth factors, angiogenic factors) that have been employed for glioma proteomics. These approaches, such as xMAP multiplex assay, enable generation of panel biomarkers that can easily be applied clinically.

In a global proteomic approach, Zhang et al. used SELDI-TOF MS and protein chip to interrogate sera from patients with astrocytoma, other brain tumors, and

healthy controls [20]. Four upregulated and three downregulated proteins were used in a decision tree classification model that achieved a sensitivity of 84.6 % and specificity of 84.4 % in detection of astrocytomas.

Other MS analysis has enabled identification of differentially abundant serum proteins. 2DE coupled with MALDI-TOF MS was applied to GBM and normal sera for protein biomarker discovery [21]. Haptoglobin α-2 was upregulated in patient samples compared to controls. To demonstrate the association of elevated serum haptoglobin with GBM, an ELISA validation was performed using different sera from patients with GBM and low-grade gliomas and normal controls, and the difference was very significant ($p = 0.0001$). Indeed, the upregulation of this protein was confirmed at the transcript level, by immunohistochemistry, and the expression was associated with tumor grade. Gautam et al. used iTRAQ-based LC-MS/MS on pooled plasma from patients and controls to identify 296 differentially expressed proteins with high certainty, of which 61 were upregulated in patient samples [22]. The authors noted that many of these proteins were associated with GBM and other cancers. However, they validated 3 biomarkers, namely, ferritin light chain (FTL), carnosinase 1 (CNDP1), and S100A9, in a test set of ten plasma samples using ELISA. The potential of these biomarkers is awaited. Of 152 peptides, clusters of *m/z* between 2 and 55 kDa were assayed in sera from GBM patients by SELDI-TOF MS, and 11 clusters (6 overexpressed and 5 underexpressed) significantly differed between patients and controls ($p < 0.05$) [23]. This technology, followed by LC-MS/MS identified S100A8, S100A9, and CXCL4 as candidate GBM biomarkers that were confirmed by ELISA. Circulating S100B, NPY, and SCGN may be early detection biomarkers of glioblastomas because their levels were markedly elevated in 2 of 3 patients one year prior to overt clinical disease manifestation [24]. As well, the prognostic potential of serum S100B was unveiled in a pilot study by Vos et al. [25]. High levels above the median value were significantly associated with shorter survival.

In a discovery phase, SELDI-TOF MS was applied to serum samples from patients with grade II astrocytomas, anaplastic astrocytomas, GBMs, and controls [26]. A specific peak identified as β-chain of α-2-Heremans-Schmid glycoprotein (AHSG) was observed to decrease in levels with increasing tumor grade, suggestive of its utility as a prognostic biomarker. This possibility was assessed in an independent sample set from GBM patients. Expectedly, shorter survival was associated with low serum levels of AHSG. A prognostic index including age, Karnofsky score, and serum AHSG levels could differentiate shorter survival patients (median survival $<$ 3 months) from those who survived longer (median survival $>$ 2 years). Elevated serum EGFR is associated with reduced OS as well ($p = 0.01$) [27].

A number of predictive protein biomarkers of GBM have been uncovered. Increased plasma MMP2, 8 h after cediranib treatment correlated with decreased PFS and OS [28]. Additionally, increased levels of PlGF and bFGF significantly correlate with longer OS. Higher PlGF and IL-8, as well as lower bFGF and sTie2 are significantly associated with radiographic response. Moreover, increased plasma levels of sVEGFR, Tek/Tie2 (sTie2) receptor, and stromal cell-derived factor 1a (SDF1a) predict radiographic disease progression. Additionally, circulating GFAP and PlGF may complement the neuroradiographic differential

diagnosis of GBM and supratentorial intracranial metastasis. Differentiation of GBM from other metastasis achieved an AUROCC of 0.73 ($p < 0.05$) [29].

Levels of cytokines, immune mediators, and angiogenic factors are altered in glioma patients. Serum Th1 cytokine, IL-12, was significantly reduced in patients with meningiomas ($p = 0.03$), anaplastic astrocytomas ($p < 0.001$), and GBM ($p = 0.001$) [30]. Serum IL-10 (Th2 cytokine), however, was significantly elevated in anaplastic astrocytomas ($p = 0.02$) and GBM ($p = 0.03$). Serum IL-6, IL-1β, TNF-α, and IL-10 were elevated three-fold, while VEGF, FGF-2, IL-8, IL-2, and GM-CSF were up two-fold in GBM patients [31], and these levels correlated with tumor grade, proliferation, and aggression. Reynes et al. observed elevated levels of immune, coagulopathy, and angiogenic proteins in plasma of GBM patients [32]. CRP, IL-6, TNFα, sialic acid, fibrinogen, endogen thrombin generation (ETG), prothrombin fragments 1 and 2, tissue factor, VEGF, sVEGFR1, and thrombospondin were all elevated; however, all inflammatory mediators (CRP, IL-6, TNFα, SA) as well as prothrombin fragments, ETG, VEGF, sVEGFR were elevated at significant levels in patient samples. The changes in cytokine profile appear useful in predicting patients who will not tolerate antiangiogenic (aflibercept) therapy [33]. Changes in IL-6, IL-10, and IL-13 levels were associated with toxicity. IL-13 change from baseline within 24 h was a surrogate for predicting endothelial dysfunction and hence patients who were toxic to treatment. Elevated IL-6 and IL-10 identified patients at risk of fatigue on these treatments.

In a large-scale study whereby serum CD14 and CD23 were measured in 1079 glioma patients and 736 healthy controls, it was demonstrated that the presence of gliomas was associated with elevated levels of CD14 and decreased CD23, and this association was strongest in GBM patients [34]. This study provided further evidence for the role of immunoregulatory molecules in gliomatogenesis.

The tyrosine–lysine–leucine (molecular weight 40) or chitinase-3-like protein 1, human cartilage glycoprotein-39 molecule (YKL40), has been extensively studied in many neoplastic conditions. This molecule causes ECM degradation and connective tissue remodeling and hence promotes cancer cell migration. Apart from tissue expression, the circulating levels of YKL40 are elevated in cancer patients, and this is associated with poor outcomes.

While it may not be specific to brain cancer, serum levels of YKL40 are significantly elevated in glioma patients, and this is associated with disease progression and poor outcome. In 343 glioma patients, serum YKL40 was measured longitudinally by ELISA and compared to disease status and patient survival [35]. In reference to radiographic disease detection, serum YKL40 was significantly lower in patients with no disease compared with those with disease (anaplastic gliomas $p = 0.008$; GBM $p = 0.0006$). Increased YKL40 was an independent predictor of worse survival (HR 1.4, $p = 0.01$ for anaplastic gliomas and HR 1.4, $p = 0.0001$ for GBM). In another study, plasma from 111 patients, 99 healthy controls, and 40 others with non-glial cell brain tumors were subjected to ELISA analysis of GFAP, YKL40, and IGFBP2 [36]. All three biomarkers were significantly elevated in patient samples than healthy controls. However, YKL40 and GFAP could differentiate patients with GBM from those with non-glial cell brain

tumors ($p = 0.04$). As a diagnostic panel to differentiate GBM from non-glial cell brain tumors, the three biomarkers achieved an AUROCC of 0.77. Plasma levels of IGFBP2 and GFAP had significant correlation with tumor volume. For their prognostic utility among GBM patients, preoperative plasma IGFBP2 was an independent predictor of OS (HR 1.3, $p = 0.05$).

18.4.6 Clinical Utility of GBM Protein Biomarkers

Several serum proteins demonstrate differential levels between GBM patients and healthy controls. While not validated, serum levels of some including YKL40, GFAP, IL-2, PIGF, PAI1, TNFα, Cathepsin-D have diagnostic potential and may be useful in multivariate index assays. Biomarkers such as elevated levels of VEGF, PAI1, IGFBP2, YKL40, S100B, and CRP are associated with prognostic outcomes of adverse PFS and OS. Elevated MMP levels (>227.5 ng/ml) before bevacizumab and irinotecan treatment predict response. Likewise, nonresponders to the combination therapy have increased plasma levels of G-CSF and IL-8 before commencement of treatment. In patients with recurrent GBM on bevacizumab and irinotecan regimen, improved PFS and OS are associated with decreases in plasma VEGF and MMP9 after treatment. Increases in MCTP3, MIF, and IP10 4 weeks after aflibercept therapy may predict disease progression, while increases in plasma IL-8 and sVEGFR1 4 weeks after cediranib therapy predict poor PFS. Thus, serum proteins hold great potential for the management of GBM patients.

18.4.7 Circulating GBM Cells

Although GBM is an aggressive high-grade tumor, it locally invades surrounding tissues with virtually no distant "soil." Together with the lack of epithelial marker expression, circulating GBM cells (CGBMCs) have not attracted intensive investigation. Indeed, some attempts have encountered difficulties and failures [37, 38]. However, using different techniques, the isolation and clinical relevance of CGBMCs have been revealed. Sullivan et al. used a microfluidic device to rather deplete hematopoietic cells from patient blood, enabling CGBMCs to be uncovered [39]. The detection rate was 39 %. These CGBMCs expressed mesenchymal rather than neural markers. The findings provide evidence that GBM shed cells into the circulation with mesenchymal characteristics and that these cells are highly migratory. Moreover, CGBMCs were found to harbor additional mutations to those of the primary tumor. Other investigators enriched for mononuclear cells and then stained for GFAP to identify CGBMCs [40]. Of the 141 patients, 29 (20.6 %) were positive for CGBMCs. To confirm that these cells were indeed CGBMCs, they demonstrated their absence in blood from healthy individuals and the presence of the GBM-signature, $7^{+}/10^{-}$ chromosomal alterations, as well as *EGFR* amplification in

CGBMCs. Macarthur et al. addressed the pertinent issue of how to isolate or detect CTCs in patients with tumors such as GBM and renal cell carcinoma that do not express epithelial adhesion cell molecules [41]. Their strategy targeted telomerase activity, which is high in almost all cancer cells. An adenoviral system was used to detect CGBMCs. While this pilot technical study was on brain tumor patients, the method is applicable to other cancers as well.

18.4.8 Circulating Endothelial Cells as GBM Biomarkers

The angiogenic and vasculogenic nature of GBM is coupled with the recruitment from the bone marrow of endothelial progenitor cells (EPCs) and the release of endothelial cells into the circulation. These circulating vascular-associated cells have been explored for their prognostic and predictive role in GBM patients.

To evaluate the possibility of using circulating endothelial progenitor cells (CEPCs) as surrogate biomarkers of tumor angiogenicity, blood from glioma patients and controls were separated into plasma and cellular fractions [42]. The plasma was used for angiogenic assay, while the cellular fraction was used to phenotype and enumerate CEPCs by their co-expression of CD133 and VEGFR2. Plasma from GBM patients scored significantly higher on the fractional angiogenic scale compared to plasma from patients with low-grade tumors and controls. CEPCs ranged from 0.1 to 1.6 % of circulating mononuclear cells. The presence of CEPCs at the time of resection correlated significantly with survival. In another analysis, CEPCs were significantly higher in GBM patients than in patients with secondary brain metastasis ($p < 0.04$) and healthy controls ($p < 0.004$) [43]. VEGF and GM-CSF involved in CEPC mobilization were also significantly higher in circulation of GBM patients compared to controls. As will be expected, GBM patients with higher CEPCs had significantly higher tumor blood vessel density than patients with low levels. CECs and CEPCs detected by CD45dim/CD34+/CD133 were significantly higher in brain tumor (GBM and meningioma) patients than controls and were even much higher in GBM than meningioma patients [44].

The elevated CECs and CEPCs may have clinical utility. Blood from newly diagnosed GBM patients on standard radiotherapy and temozolomide treatment were collected before and at the end of therapy for analysis [45]. Pretreatment CECs and microparticles were significantly higher in patients than healthy controls ($p < 0.001$), and these were associated with procoagulant state as detected by significant decrease in endogen thrombin generation time and by an increased phospholipid-dependent clotting time. High pretreatment CECs were associated with poor OS. Similarly, *MGMT* methylation that was detected in 27 % of GBM patients was associated with higher OS (66 vs. 30 weeks, $p < 0.004$). Recurrent GBM patients on bevacizumab and/or irinotecan treatment had blood samples evaluated for CD109+ tumor endothelial cells and other CEC/CEPC subtypes assayed by 6-color flow cytometry [46]. Noted findings were that patients with baseline CECs > 41.1/ml of blood had increased PFS (20 vs. 9 weeks, $p = 0.008$)

and OS (32 vs. 23 weeks, $p = 0.03$). This was confirmed in a separate data set of patients on bevacizumab alone therapy. Treated patients who were free of MRI progression after 2 months demonstrated significantly decreased CECs.

18.5 GBM Extracellular Vesicles

GBM-derived EVs contain cargo of tumor origin, including structural transcripts, noncoding RNAs, and proteins. These molecules have been explored as potential biomarkers for patient management. Apart from being loaded with diagnostic, prognostic, and predictive biomarkers, the study of EVs also shed some light on tumor biology, which is important in disease management.

Established GBM tissue biomarkers such as *IDH1/2* and *EGFRvIII* mutations; proteins including podoplanin, EGFR, EGFRvIII, and IDH1; and miRNAs are enriched in circulating and CSF EVs. Skog et al. were able to detect the *EGFRvIII* mutation in GBM-derived exosomes from five patients who also had similar mutations in tumor tissue samples and in two patients with biopsy negative for this variant [47]. Using highly sensitive PCR platform targeting *IDH1R132H* mutation, Chen et al. failed to detect this mutation in sera from patients with the mutation in tissue samples [48]. However, CSFs were positive in 5/8 patients. The levels of *IDH1* mutant transcripts correlated with tumor burden in regard to volume.

MiRNAs are enriched in circulating EVs from GBM patients. Eleven miRNAs including the tumor-associated miR-21 were found enriched in circulating EVs from GBM patients. While the levels of miRNAs were lower in microvesicles than in tumor tissue samples, they showed a good correlation [47]. CSF from GBM patients also reveals significantly high (ten-fold higher than controls) miR-21 in EVs. Their levels decreased following tumor removal, and miR-21 was very accurate in differentiating patients from controls [49]. Using PCR-based TagMan Low-Density Arrays, followed by qRT-PCR validation of serum microvesicles from GBM patients and controls, a small noncoding RNA (RNU6-1), miR-320, and miR-574-3p were significantly associated with diagnosis of GBM. However, RNU6-1 was a stable independent diagnostic biomarker of GBM [50].

Proteomic approaches have enabled measurement of proteins in EVs isolated from sera of GBM patients. Using a microfluidic chip technology to label EVs by magnetic nanoparticles followed by EV analysis with micro-nuclear magnetic resonance, EGFRvIII, EGFR, IDH1R132H, and podoplanin were measured in EVs from GBM patients and healthy control sera [51]. Importantly, the presence of high circulating levels of these proteins in EVs significantly predicted temozolomide and radiation treatment failure.

Cancer-derived EVs are important in tumor biology as well. Skog et al. demonstrated that GBM-derived exosomes deliver mRNA to microvesicular endothelial cells in which these messages were translated [47]. Importantly, these exosomes were enriched for angiogenic proteins that stimulated tubule formation by endothelial cells. GBM microvesicles also induced GBM cell proliferation. Increased

EVs in CSF of glioma patients are associated with procoagulant activity and thromboembolic phenomenon [52]. EVs from CSF (but not from patient blood) stimulated endothelial cell proliferation and migration via AKT and/or β-catenin signaling.

18.6 Summary

- The commonest nervous system tumors are gliomas.
- High-grade gliomas, known as glioblastomas (GBMs), are associated with a poor survival outcome.
- The molecular pathology of GBM includes epigenetic and genetic alterations that alter oncogenic signaling pathways such as the RTK/MAK, RTK/PI3K, the cell cycle, and other growth factor signaling pathways.
- Circulating GBM biomarkers include specific gene promoter methylation (e.g., *MGMT* and *CDKN2A*), LOH (e.g., 1p, 10q), mutations (e.g., *IDH1*/*IDH2*), and the plethora of serum proteins.
- Emerging GBM biomarkers include circulating miRNA, GBM cells, endothelial cells, and extracellular vesicles.

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