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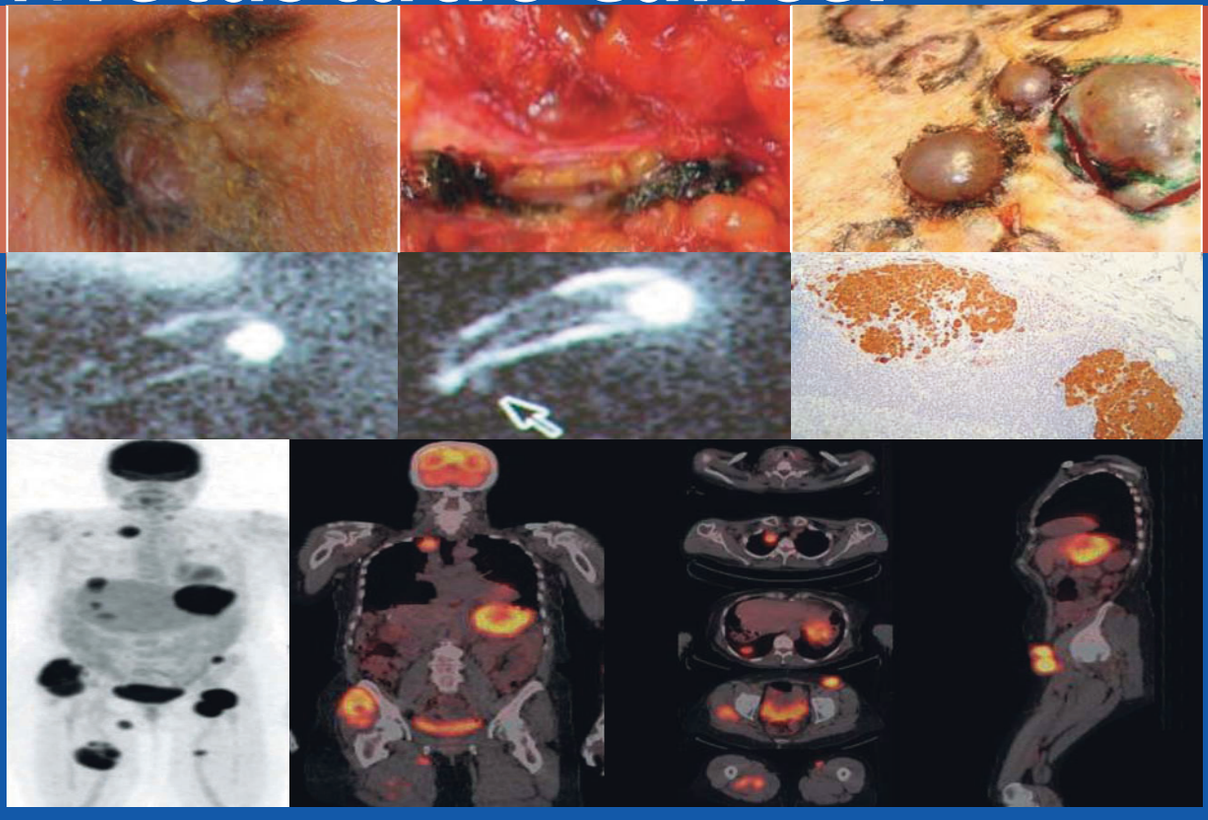
Editor

From Local Invasion to Metastatic Cancer



Involvement of Distant Sites Through the Lymphovascular System

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From Local Invasion to Metastatic Cancer

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We would like to thank the staff at Humana Press/Springer, Paul Dolgert, Frances Louie and Frank McGuckin, for their advice and expertise in making this book possible.

Foreword

This textbook captures the highlights of the timely symposium on cancer metastasis and the lymphovascular system. The symposium provided a unique forum for the convergence of biomedical research breakthroughs, presented by basic scientists, surgeons, radiologists, immunologists, and others, to address the central role of the lymphovascular system in the spread of cancer. The perspectives offered by experts in their respective fields of scientific inquiry inspired cross fertilization of ideas and paradigm-shifting hypotheses.

Regional lymph node metastasis is considered a prognostic indicator for the aggressive potential of solid tumors. However, despite recent advances in cancer biology and experimental therapeutics, critical gaps remain in our understanding of the molecular regulation of lymph node metastasis involving lymphatic invasion as a key feature. Lymphatic vascular biology has earned a prominent position in the field of cancer research, and lymphangiogenesis appears to be an attractive new target in the war against cancer.

The panel of experts assembled for the symposium reviewed the current status of basic, clinical and translational research in the field, including model systems to study lymphangiogenesis and angiogenesis; molecular imaging of lymph nodes; and therapeutic targeting of the lymphovascular system. The confluence of new knowledge and ideas was supported by a presentation on the vision of the National Cancer Institute. Perhaps no other time in the history of medicine has there been a strategic alignment of breakthroughs in biology, technology, and clinical research to advance the field in cancer metastasis and the lymphovascular system.

Preface

The aim of this book is to trace cancer metastasis from the primary sites to the regional lymph nodes and distant organs through the mechanism of local proliferation resulting in metastasis through the lymphovascular system. Rational therapy may be developed to curb the process of metastasis upon understanding these crucial steps of metastasis. Whether the cancer cells utilize the lymphatic or vascular channels or both to metastasize will be examined.

This book summarizes the *2nd International Symposium on Cancer Metastasis: Basis for Rational Therapy* being held in San Francisco from May 3–5, 2007 by bringing together the basic scientists and clinicians to ask the central question of the role of the lymphovascular system in the spread of cancer. Thus, this book is able to link the bench to the bedside and vice versa in understanding the mechanisms of cancer metastasis.

In human solid cancers, the nodal status is the most important prognostic indicator for patients' outcome. Recent developments in the sentinel lymph node (SLN) concept and technology have resulted in the application of such a procedure to define the first draining node or SLN as the primary gateway through which the cancer will spread.

Part I addresses several important developments in the biology and clinical aspects of cancer metastasis. Part II describes the relationship between tumor microenvironment and proliferation. Part III defines the process of lymphangiogenesis and angiogenesis with special reference to cancer metastasis. Part IV summarizes the development of multiple approaches in the imaging of cancer during its course of metastasis. Part V attempts to use the lymphatic system as a target to treat cancer. Part VI updates the latest cellular and molecular mechanisms of cancer metastasis. Part VII examines the role of molecular targeted therapy against growth factor receptors, signaling pathways and angiogenesis as therapeutic targets. Part VIII emphasizes the impact of tumor burden in the sentinel lymph nodes on the clinical outcome in several solid cancers. Part IX defines immune responses in the draining lymph nodes against cancer relating to immunotherapy against cancer. The role of cancer stem cells is being explored in Part X. With advent of molecular techniques, the genomic signatures of cancer may be developed and analyzed in Part XI. Parts XII and XIII summarize the therapeutic results of using new approaches in cancer treatment. Any promising leads from clinical trials in metastatic cancer may be used in the future as adjuvant therapies for occult metastatic deposits. Part XIV poses unanswered questions as future perspectives.

Perhaps, more uniquely, this book will bring the basic scientists, radiologists and clinicians together resulting in cross fertilization between these disciplines with intention to develop strategies to curb the process of metastasis.

San Francisco, CA — Stanley P. L. Leong, M.D.

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Color Plates

COLOR PLATE 1 Models of metastasis and gene expression profiles. Different theories of metastasis result in different outcomes with regard to gene expression profiles. In the conventional and stem cell models, **a** & **b**, the differential gene expression is thought to come from the specific mutations that lead to the development of cancer (*black bars*). In the conventional model (**a**), the mutated tumor cell gives rise to a cancer. A subsequent mutation, *dark cell*, confers metastatic ability. The differential gene expression profile of the metastasis reflects the genetic changes in the metastatic cell, which are now conferred to its progeny. In the stem cell theory of metastasis (**b**), the initial oncogenic mutation is responsible for alternations in gene expression. White cells are differentiated progeny of stem cell, but no further mutation is necessary to confer metastatic ability as it already exists in the cancer stem cell. Thus, no additional mutations are reflected in the gene expression profile. In the germline theory (**c**), the expression profile comes from the oncogenic mutations (*black bar*) as well as the genetic background (*white*). Regardless of what further oncogenic mutations, the genetic background of the host dominates the gene expression signature, leading to similar gene expression profiles in the primary and metastatic tumors. (Chapter 1, Fig. 2; *see* discussion on p. 6)

COLOR PLATE 2 Sentinel node with micrometastasis in gastric cancer. (Chapter 4, Fig. 1; *see* discussion on p. 30)

COLOR PLATE 3 Patterns of metastasis in sentinel nodes. **a.** Marginal sinus type, **b.** Intermediate sinus type, **c.** Parenchymal type, **d.** Diffuse type. (Chapter 4, Fig. 3; *see* discussion on p. 35)

COLOR PLATE 4 Proliferative activity of metastatic foci. **a**. Metastasis, **b**. Micrometastasis, **c**. Isolated tumor cell, **d**. Single cell. (Chapter 4, Fig. 4; *see* discussion on p. 38)

COLOR PLATE 5 Tracing of lymphatics of both hemi upper torsos (Male: **A** and **C**, Female: **B** and **D**) retrogradely from each first echelon lymph node colour coded. (Chapter 5, Fig. 8; *see* discussion on p. 51 and complete caption on p. 51)

COLOR PLATE 6 Signaling molecules possibly involved in tumor-induced lymphangiogenesis. In this figure, several growth factor and cytokine receptors and their

ligands are depicted that have been implicated in lymphangiogenesis and are expressed in the context of tumors. (Chapter 9, Fig. 1; *see* discussion on p. 97 and complete caption on p. 96)

COLOR PLATE 7 Tumor–vessel interactions: local and distant effects. Primary tumors secrete growth factors that can act locally on the lymphatics to increase vessel diameter and to increase the number of vessels. (Chapter 9, Fig. 2; *see* discussion on p. 98 and complete caption on p. 99)

COLOR PLATE 8 Chronological examination of 3D-CT LG *(14)*. 3D-CT LG was examined 1, 3, and 5 min after iopamidol injection. Iopamidol flowed to extend over the SN into the next nodes. Five bead-like grouped lymph nodes in the axilla can be visualized by partially removing the pectoral muscle in the CT monitor. These are thought to be the order of lymph metastasis. *Arrows* point to lymph nodes 1–5 after SN. (Chapter 14, Fig. 3; *see* discussion on p. 164)

COLOR PLATE 9 Detection of SN metastasis *(14)*. (**a**) Three SNs are recognized by 3D-CT LG. The right node drained from the main lymph duct was not metastasized. On the other hand, the other two nodes drained from the narrow collateral duct were metastasized. Dye and isotope could not reach as such the latter collateral nodes. These might become false-negative study. Therefore, 3D-CT LG is effective in raising accuracy of SNB. (**b**) The SN was metastasized, but the second node and the other nodes were all negative. (Chapter 14, Fig. 4; *see* discussion on p. 165)

COLOR PLATE 10 Image quality analysis using three different reconstruction softwares: filtering back projection (FBP), iterative reconstruction without attenuation correction (IRNC), and iterative reconstruction with attenuation correction (IRAC). *White arrows*: liver metastases nondetected by FBP reconstruction. (Chapter 15, Fig. 1; *see* discussion on p. 171)

COLOR PLATE 11 SRS whole-body scan (*left*) in a patient with metastatic thymic neuroendocrine tumor demonstrated several foci of increased ^{111}In-pentetreotide uptake in the chest, and right upper and lower abdomen. Selected coronal and sagittal SPET/CT slices showed the precise localization of one of the areas of abnormal uptake in the right lower abdomen (**A**) and in the right upper abdomen (**B**). Fused images (*right*) enabled metastatic lesions. (Chapter 15, Fig. 2; *see* discussion on p. 173)

COLOR PLATE 12 Lymphotropic properties of Ad. A transmission electron micrograph image of human adenoviruses depicting the icosahedron-shaped viral particles (**a**). Alexa fluor-555 labeled AdTSTA-FL virus was injected into the forepaw of severe combined immunodeficiency (SCID) mice and confocal images of lymph node (LN) sections taken at 1 or 24 hours after viral injection revealed the presence of fluorescent virus in the draining axillary node (**b**). Functional lymphatic drainage of adenovirus from the forepaw (**c**) or the subcutaneous (s.c.) chest site (**d**) to the ipsilateral draining LN can be detected by bioluminescent luciferase

signals. 1×10^7 infectious units of constitutive CMV promoter-driven virus (Ad–CMV–FL) were injected into the nontumor-bearing SCID mice. (Chapter 16, Fig. 1; *see* discussion on p. 181)

COLOR PLATE 13 Prostate-specific TSTA system and imaging reporter Ad. A schematic representation of the two-step transcriptional amplification (TSTA) system (**a**). The AdTSTA-FL and the luciferase bioluminescent signals produced by this Ad after intratumoral injection as imaged by luciferin-based bioluminescence imaging (BLI) (**b**). AdTSTA-sr39tk PET-imaging reporter vector and the ^{18}F-FHBG signal produced after intratumoral vector injection (**c**). PSE-BC = chimeric PSA enhancer/promoter; GAL4/VP2 = Fusion of the yeast GAL4 DNA-binding domain and two copies of the herpes simplex virus VP16 activation domain; TATA = TATA box; 5× GAL4 = 5 GAL4-binding sites; and HSV1-sr39tk = mutant herpes simplex virus type 1 thymidine kinase. (Chapter 16, Fig. 2; *see* discussion on p. 181)

COLOR PLATE 14 Functional imaging of LN metastasis. Bioluminescence imaging of a male SCID mouse bearing LAPC-9 prostate tumor on left upper back (**a**). BLI was performed 4 days after injecting 1×10^7 infectious units of AdTSTA-FL into the left paw. Histological confirmation of metastatic prostate tumor cells in the LN by H&E (left, 10×) and anticytokeratin staining (right, 40×) (**b**). Transverse and coronal sections of 18F-FHBG PET from another LN-positive mouse scanned 4 days after injecting 1×10^8 infectious units of AdTSTA-sr39tk into the left paw (**c**). Photograph of enlarged brachial and axillary LNs (**d**). (Chapter 16, Fig. 3; *see* discussion on p. 181)

COLOR PLATE 15 (**A**) Malignant melanoma of the heel with popliteal SLN metastasis. (**B**) The cancer cells reach the regional nodes by "embolization" (*arrows*) rather than by centripetal permeation, discontinuous embolism *(10,22,62,93)*. (**C**) *Arrow* indicates metastatic embolus. (Chapter 30, Fig. 1; *see* discussion on p. 351)

COLOR PLATE 16 (**A**) Lymphangiogram showing the lymphatic drainage of the right testicle (AP view) (dog injection). (**B**) Lateral view. Direct lymphangiogram of the testicle disclose the SLN in the precaval area (dog). (**C**) Bilateral direct testicular lymphangiogram in human concomitant with dorsum of the foot injection. The *arrows* indicate the location of the SLN in the *right* side as well as the *left*. (Chapter 30, Fig. 2; *see* discussion on p. 351)

COLOR PLATE 17 (**A**) *Arrows* indicate SLN harboring metastasis (lateral view). (**B**) *Arrows* indicate SLN metastasis (AP view). (**C**) Surgical specimen of retroperitoneal lymph node dissection. *Arrows* indicate SLN. (Chapter 30, Fig. 3; *see* discussion on p. 351)

COLOR PLATE 18 (**A**) Advanced malignant neoplasia of the testicle (reluctant patient). (**B**) Metastasis lymph nodes pre-aorta–cava area. (**C**) SLN of the testicle harboring metastasis. (Chapter 30, Fig. 4; *see* discussion on p. 351)

COLOR PLATE 19 Lymphazurin 1% is being injected in the subserosal layer of right colon. (Chapter 31, Fig. 1; *see* discussion on p. 366)

COLOR PLATE 20 Blue lymphatic traveling to a blue sentinel lymph node (*arrow*) seen on the retroperitoneal surface. (Chapter 31, Fig. 2; *see* discussion on p. 366)

COLOR PLATE 21 Fluorescent lymphatic reaching a fluorescent sentinel lymph node (*arrow*) seen in a dark room with Wood's Light illumination. (Chapter 31, Fig. 3; *see* discussion on p. 368)

COLOR PLATE 22 IHC staining with antibody to S-100 demonstrates significant reduction in IDC area and density in SN versus non-SN (NSN). T cell (CD43) area was also reduced in SN compared to non-SN while B cell (CD20) area was not altered. The presence (SN+) or absence (SN–) of metastases to the SN did not appear to alter the differences in IDC area or density. (Chapter 35, Fig. 1; *see* discussion on p. 413)

COLOR PLATE 23 Sample amplification plot for GAPDH standards demonstrating the threshold cycle (C_t) for each of the serial dilutions. In the second graph, the C_t's are plotted against the Log(quantity—C0), providing a standard line with a slope and quality of fit. (Chapter 35, Fig. 5; *see* discussion on p. 416)

COLOR PLATE 24 Significant difference in cytokine gene expression in the SN compared to the NSN (non-SN). The relative patterns of cytokine expression are reversed in the presence or in absence of residual melanoma found during LM/SL. Presence of residual tumor significantly increases the levels of cytokine expression in the SN. Most dramatic increase is noted in the expression of IDO, which is an enzyme expressed in immune downregulatory dendritic cells *(56,57)*. (Chapter 35, Fig. 6; *see* discussion on p. 417)

COLOR PLATE 25 Real-time quantitative PCR amplification plot for GAPDH, a standard housekeeping gene is shown. The RNA was extracted from an archival tissue that was fixed in formalin and embedded in paraffin. The figure demonstrates successful quantitative measurement of GAPDH gene expression in such tissue sample. (Chapter 35, Fig. 7; *see* discussion on p. 419)

COLOR PLATE 26 Relative T cell and B cell area and IDC density in SN based on preoperative dose of GM-CSF. Patients receiving GM-CSF 150 $\mu g/m^2$ appeared to have the greatest morphologic change to the SN. (Chapter 35, Fig. 9; *see* discussion on p. 420)

COLOR PLATE 27 Association between Br-CSC gene expression profiles and prognosis of breast cancer patients. A Pearson correlation coefficient was calculated for the correlation between a gene signature obtained from Br-CSCs (the "invasiveness gene signature" or IGS) and each of the 295 tumors included in a publicly available breast cancer patient database (the

Netherlands Cancer Institute database) on the basis of the expression values of the 186 genes that are included in the gene signature. Patients were separated into two groups according to the correlation values, with 0 used as the threshold. Kaplan–Meier survival curves for the two groups were compared, with overall survival (Panel **A**) and metastasis-free survival (Panel **B**) as the clinical end points. Patients with tumors with a gene expression pattern that was similar to the IGS (correlation coefficient, >0) had worse outcomes than those with tumors with a gene expression pattern that was not similar to the IGS (correlation coefficient,≤0). Reproduced with permission from Liu et al., *N Engl J Med*, 2007; 356:217–226. Copyright © *2007 Massachusetts Medical Society. All rights reserved.* (Chapter 38, Fig. 1; *see* discussion on p. 446)

COLOR PLATE 28 Metastasis-free survival of breast cancer patients after stratification based on the combined use of the IGS and the WR signature. A Pearson correlation coefficient was calculated for the correlation between each of the two signatures (IGS and WR) and each of the 295 tumors included in the Netherlands Cancer Institute database. Group 1 included patients with a negative correlation to both the IGS and the WR signature. Group 2 included patients with a positive correlation to either the IGS or the WR signature. Group 3 included patients with a positive correlation to both the IGS and the WR signature. The 10-year metastasis-free survival of the three groups was 80, 69, and 47%, respectively. Reproduced with permission from Liu et al., *N Engl J Med*, 2007; 356:217–226. Copyright © *2007 Massachusetts Medical Society. All rights reserved.* (Chapter 38, Fig. 2; *see* discussion on p. 446)

COLOR PLATE 29 Comparison of ER antibodies SP1 and 1D5. The SP1+/1D5– cases have cumulative survival at 10 years similar to those of SP1+/1D5+ tumors *(6)*. (Chapter 40, Fig. 5; *see* discussion on p. 472)

COLOR PLATE 30 This is an example of the hybridization of reference DNA (*green*) and tumor DNA (*red*) to an interphase spread of chromosomes (ready for mitosis and frozen by adding colchicine to stop spindle apparatus). Above the *red* line shows amplification of tumor gene copy and below the *green* line shows loss of gene copy number. (Chapter 40, Fig. 9; *see* discussion on p. 478)

COLOR PLATE 31 QRT-PRC. The RNA is extracted, reverse transcribed and mixed with two specific primers (generally within 100 bp of each other in the case of formalin-fixed tissue) and a fluorescently labeled probe specific for the-same target. The probe has both a fluorescent reporter attached to one end, and a quencher on the other—this quenches the fluorescent signal. The primers and probe anneal to the specific target gene. Due to the 5′-exonuclease activity of the Taq polymerase, already-bound fluorescent-labeled probe is degraded while new PCR product is being synthesized. This process releases the fluorescent tag from the quencher and generates a fluorescence signal that is directly proportional to the amount

of PCR product in the tube. This signal can be quantitated and the amount of mRNA determined. (Chapter 40, Fig. 10; *see* discussion on p. 479)

COLOR PLATE 32 Gene expression patterns of 85 experimental samples representing 78 carcinomas, three benign tumors, and four normal tissues, analyzed by hierarchical clustering. The closer samples are together, the more similar are their expression profiles. The tumor specimens were divided into six subtypes based on differences in gene expression. Note differences in ER– and ER+. The cluster dendrogram shows the six subtypes of tumors from *left* to *right* (colored as): basal-like, *red*; ERBB21, *pink*; normal breast-like, *green*; luminal subtype C, *light blue*; luminal subtype B, *yellow*; luminal subtype A, *dark blue(57)*. (Chapter 40, Fig. 11; *see* discussion on p. 481)

COLOR PLATE 33 Individual dendrogram branches are colored according to the strongest correlation of the corresponding tumor with the subtype centroid as defined for the Perou/Sorlie samples. The best discrimination was between the tumors of the luminal A cluster at high levels and tumors that exhibited expression profiles characteristic of the basal, HER2 or luminal B subtypes. The strongest correlation was with the basal subtype (*red branches*)—all of which are contained within the *left* branch of the dendrogram in a tight cluster. (Chapter 40, Fig. 12; *see* discussion on p. 481)

COLOR PLATE 34 Kaplan-Meier analysis of disease outcome in two patient cohorts. (**A**) time to development of distant metastasis in the 97 cases from van 't Veer et al. Patients were stratified according to the subtypes as shown in Fig. 14. (**B**) Overall survival for 72 patients with locally advanced breast cancer in the Norway cohort. The normal-like tumor subgroup was omitted in both analyses. (Chapter 40, Fig. 13; *see* discussion on p. 482)

COLOR PLATE 35 Supervised classification on prognosis signatures. (**A**) Prognostic reporter genes identify optimally two types of disease outcome from 78 sporadic breast tumors into a poor prognosis and good prognosis group. Each row represents a tumor and each column a gene. Genes are ordered according to their correlation coefficient with the two prognostic groups. The *solid line* represents the prognostic classifier with optimal accuracy; *dashed line* with optimized sensitivity. Above the *dashed line* are patients with a good prognosis and below those with a poor prognosis. The metastasis status for each patient is shown in the *right panel*: white indicates patients with metastases within 5 years and black indicates those disease free for at least 5 years. (Chapter 40, Fig. 14; *see* discussion on p. 485)

COLOR PLATE 36 Kaplan–Meier analysis of the probability that patient would remain free of distant metastases and the probability of overall survival among all patients. (Chapter 40, Fig. 15; *see* discussion on p. 487)

COLOR PLATE 37 Kaplan–Meier analysis of the probability that lymph node-negative patient (*left two panels*) and lymph node-positive patients (*right two panels*) would remain free of distant metastases and the probability of OS, respectively. (Chapter 40, Fig. 16; *see* discussion on p. 487)

COLOR PLATE 38 The recurrence score (RS) is used to predict patient prognosis, it ranges from 0 to 100 and it is divided into three risk groups: (1) a low-risk score correlating with a low risk of distant recurrence, RS 0–18; (2) an intermediate risk score correlating with an intermediate risk of distant recurrence, $18 < RS < 31$; and (3) a high-risk score correlating with a high risk of distant recurrence, $RS \geq 31$. (Chapter 40, Fig. 17; *see* discussion on p. 490)

COLOR PLATE 39 Microarray analysis of the radial and vertical growth phases within a single, large, primary melanoma. Note that in the vertical growth phase, there is a predominance of *green* signals, indicating losses of gene expression. (Chapter 41, Fig. 2; *see* discussion on p. 500)

COLOR PLATE 40 Representative primary melanoma with evaluable radial and vertical growth phases immunostained for MMP-10 (**a**) and CDH3 (**b**). Prominent staining is noted within the radial growth phase consistent with gene profile analysis showing a loss of these genes in the vertical growth phase. (Chapter 41, Fig. 3; *see* discussion on p. 501)

COLOR PLATE 41 Immunohistochemical staining for a patient with a melanoma in situ with extensive regression. The radial growth phase markers, CDH3 and MMP-10, were detected in both the primary melanoma and in the metastasis. (Chapter 41, Fig. 4; *see* discussion on p. 502)

COLOR PLATE 42 Unsupervised hierarchial clustering of metastatic melanoma defines subtypes I and II. (Chapter 41, Fig. 5; *see* discussion on p. 502)

COLOR PLATE 43 Microanalysis is able to distinguish nevi from melanoma. (Chapter 41, Fig. 6; *see* discussion on p. 503)

COLOR PLATE 44 Primary melanoma to metastasis: radial growth phase melanomas have the potential to metastasize and the development of a vertical growth phase may not be a prerequisite for the development of metastatic disease. (Chapter 41, Fig. 7; *see* discussion on p. 506)

COLOR PLATE 45 Interferon (IFN) signal transduction. IFNs bind to specific receptors and induce tyrosine phosphorylation of receptors by JAK kinases providing a docking site for the STAT proteins which are subsequently phosphorylated by the Jak kinases. Activated STAT proteins dimerize and translocate into the nucleus in order to directly bind to specific DNA sequences as transcription factors (from *[11]*). (Chapter 46, Fig. 1; *see* discussion on p. 556)

I Innovation and Experience

1 Frontiers of Cancer Research: The Metastasis Challenge

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CONTENTS

ABSTRACT

Greater than 90% of deaths in cancer patients are due to the metastatic spread of the tumor to vital organs, emphasizing the importance of efforts toward the prevention and treatment of this phenomenon. The ability to block the process of metastasis could have a significant impact on the mortality rates associated with malignancy. As a result, the study of metastasis has been a focus at numerous centers of research, including the National Cancer Institute (NCI). A plethora of new technologies developed over the last decade, including gene expression profiling, have allowed for the identification of a number of genes, many of them strictly confined to the stromal cells associated with the tumor that appear to be significantly involved in the metastatic process. This allows us to enhance our efforts at designing stromal directed therapies to be utilized in combination with more traditional regimens directed at tumor cells.

This highly personalized signature of the tumor and tumor microenvironment provides the unprecedented opportunity to develop therapeutic solutions which are also designed to be preventive, in terms of the biologic processes that support development of tumor metastasis. In addition to new therapies, genetic profiling has also led to new ways of thinking about the origins and development of metastasis and metastatic potential. Research at the NCI provides an interesting perspective on inborn genetic traits that predispose an individual to metastasis, should a malignancy develop. The presence of these genetic traits has enormous implications for prophylactic therapy. Investigations such as these continually expand the frontiers of cancer research and hold the potential to contribute to the prevention and control of metastasis and its associated mortality.

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The complexity of the group of diseases collectively called cancer arises from genomic alterations to cells during their lifespan. Deletions of tumor suppressor genes, amplifications of oncogenes, aneuploidy, translocations leading to altered gene expression, point mutations, and epigenetic changes all play a role in oncogenic transformation. These changes in turn lead to myriad downstream perturbations, all of which combine to enable the survival and propagation of malignant growth. These genetic alterations, though varying in origin, can be distilled into a few critical mechanisms. Hanahan and Weinberg identified six acquired functional capabilities in cancer: self sufficiency in growth signals (autocrine signaling); limitless replicative potential; insensitivity to antigrowth signals; evading apoptosis; sustained angiogenesis; and invasion and metastases *(1)*. To this, a seventh alteration, the ability of cells to evade detection by the immune system, can be added. The review that follows focuses on the ability of these cells to invade and metastasize.

Metastasis, which is responsible for greater than 90% of cancer deaths *(2)*, is a systemic, rather than local, disease. With our current therapies we are often able to manage locally circumscribed tumors; however, the unpredictability and inaccessibility of metastatic cancer confounds the same strategies that have been successful in helping us to control localized neoplastic disease. To make inroads against metastasis, we have to first understand the multiplicity of processes that lead to the spread of cancer and then we have to utilize this knowledge for prevention of this phase of cancer progression.

Metastasis involves a number of different steps, each critical to the eventual outcome of tumor dissemination throughout the body: cells of the primary tumor must acquire the ability to invade past the basement membrane, to migrate through the stroma surrounding the tumor, and to invade into blood vessels and lymphatic channels. After being disseminated through the circulatory system, these cells must leave the circulation within target organs (extravasation), invade the stroma, and establish micrometastatic sites. Subsequent cell proliferation and recruitment of supportive structures, such as tumor vessels (angiogenesis), lead to growth of detectable tumors (macrometastases). These various steps provide several opportunities to target the process of metastasis. The various organ systems involved in each step (to be discussed later) illustrate the systemic nature of cancer. Therefore, it is likely that our best opportunity for success will come from a multiintervention approach rather than a single target approach.

Currently there are three important factors influencing the process of metastasis that hold promise for its prevention and eventual treatment. The influences of the tumor microenvironment, the presence of cancer stem/initiating cells, and the possibility of germline inheritance of metastatic potential all merit further investigation. These factors, in concert, can have a profound influence on metastasis (Fig. 1) and are being explored as potential targets.

1. PREDICTING METASTASIS

In order to prevent metastasis, we need to be able to predict it with accuracy. Up until the last decade, our best prognosticators for metastatic potential were clinical and pathologic factors. More recently, gene expression profiling has been accepted as an approach to determine metastatic risk. In breast cancer, two independent gene expression signatures with 70 and 76 genes, respectively, have been used as prognosticators of metastasis. Both signatures have >90% sensitivity in predicting metastasis with specificity ranging from 48 to 73%, although there is

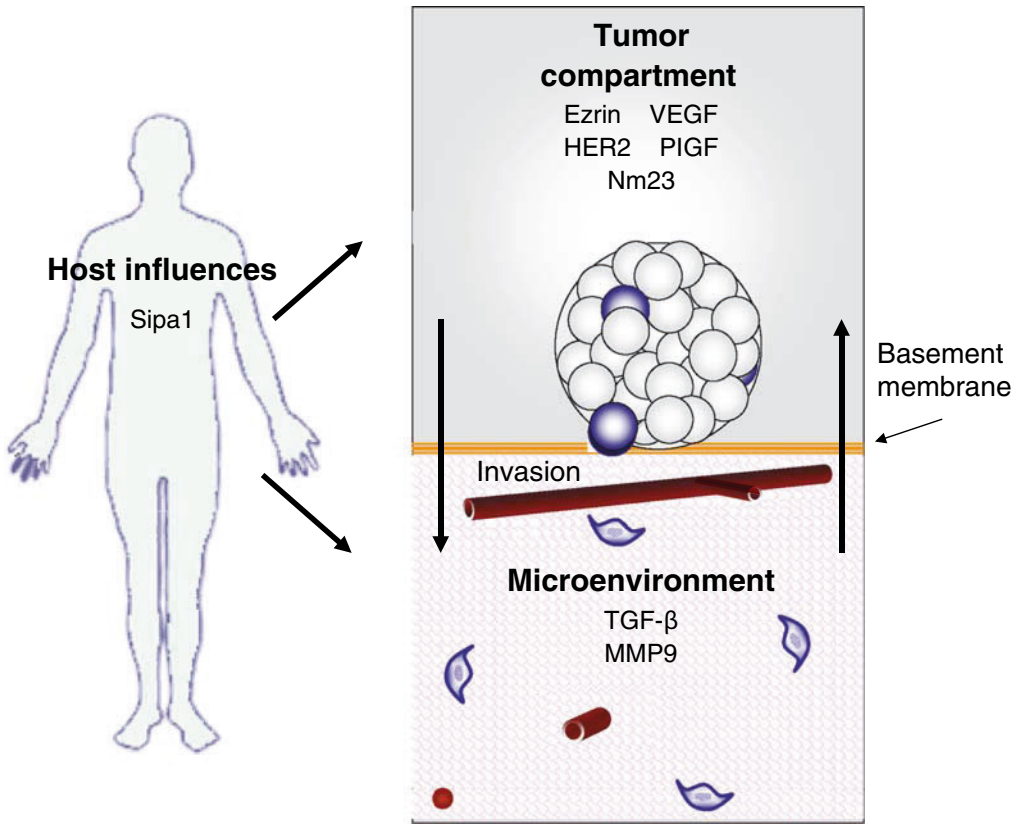

Fig. 1. Multiple factors combine to lead to invasion and metastasis. Factors can derive from the tumor cells themselves, from the cells of the tumor microenvironment, or from the host genetic background. Here the crosstalk between the factors results in the invasion of a tumor-initiating cell (*shaded*) through the basement membrane.

little overlap between the two signatures *(3)*. The 70 gene signature proved to be superior to classical prognostic criteria *(4,5)*—age of patient, tumor size, axillary lymph node status, histological grade, and steroid receptor expression—in predicting metastases. For example, 50% of the patients classified as high-risk patients by gene profiling using this signature eventually developed metastatic disease, whereas only 20–25% of patients classified as high risk by the National Institute of Health (NIH) and St. Gallen criteria went on to develop metastases. By utilizing the molecular signature, unnecessary adjuvant therapy can be avoided in a significant number of patients. New approaches, such as whole genome scanning and actual tumor sequencing, promise even more specificity for this process of risk assessment.

2. ORIGINS OF METASTATIC POTENTIAL

Aside from being a tool in therapeutic decision-making, gene expression signatures have informed our understanding of the risk of metastasis. The conventional model of metastasis hypothesizes that metastatic potential develops as a result of the somatic mutations that induce heterogeneity in a tumor, including a metastatic phenotype *(6)*. These mutations are thought to occur relatively late in the process of tumorigenesis, certainly after the oncogenic event. It has been proposed that these mutations lead to the development of a subpopulation of cells that have

the ability to metastasize. Therefore, it would be expected that the tumor cells in a metastatic lesion would all have the ability to metastasize in comparison with that in the primary lesion in which only a small subset of the cell population has the ability to spread to distant sites (Fig. 2a

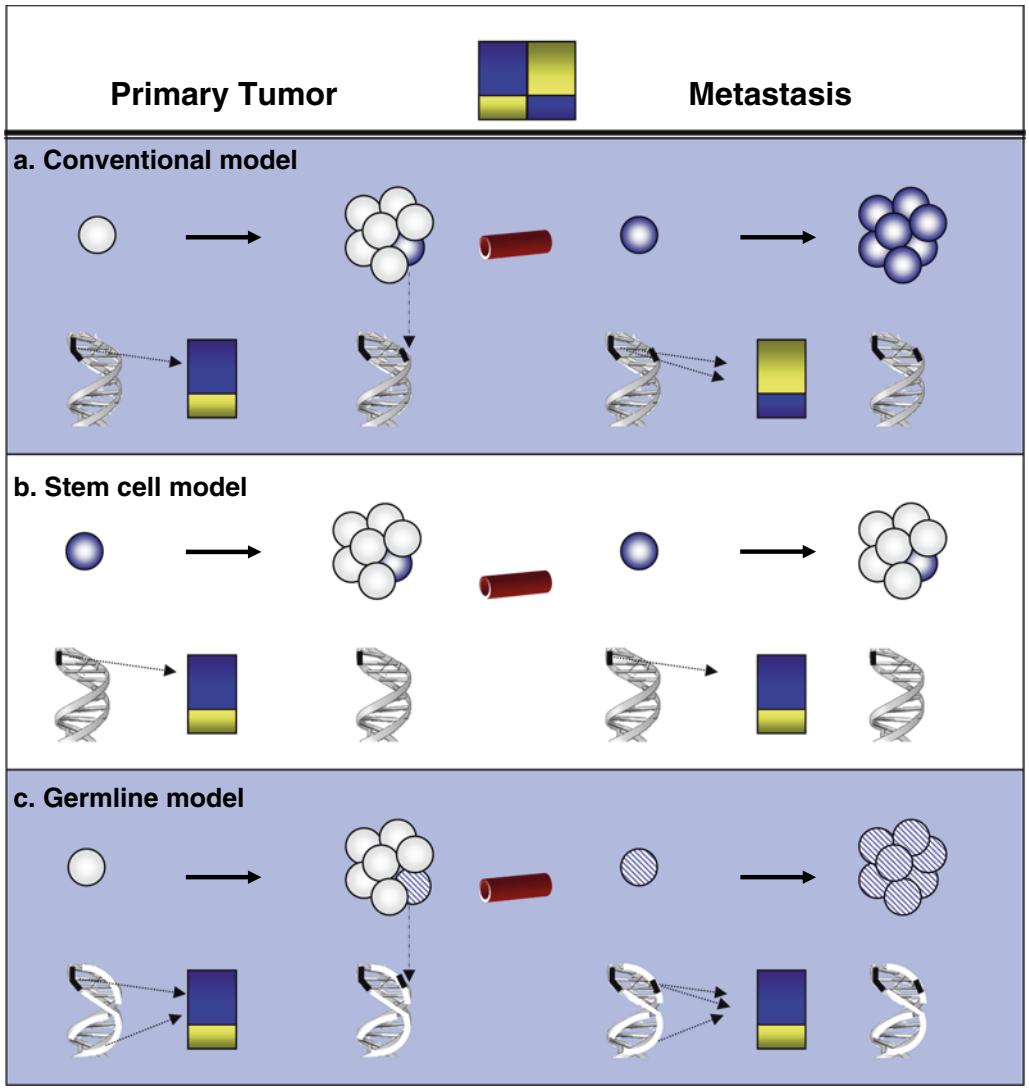

Fig. 2. Models of metastasis and gene expression profiles. Different theories of metastasis result in different outcomes with regard to gene expression profiles. In the conventional and stem cell models, **a** & **b**, the differential gene expression is thought to come from the specific mutations that lead to the development of cancer (*black bars*). In the conventional model (**a**), the mutated tumor cell gives rise to a cancer. A subsequent mutation, *dark cell*, confers metastatic ability. The differential gene expression profile of the metastasis reflects the genetic changes in the metastatic cell, which are now conferred to its progeny. In the stem cell theory of metastasis (**b**), the initial oncogenic mutation is responsible for alternations in gene expression. White cells are differentiated progeny of stem cell, but no further mutation is necessary to confer metastatic ability as it already exists in the cancer stem cell. Thus, no additional mutations are reflected in the gene expression profile. In the germline theory (**c**), the expression profile comes from the oncogenic mutations (*black bar*) as well as the genetic background (*white*). Regardless of what further oncogenic mutations, the genetic background of the host dominates the gene expression signature, leading to similar gene expression profiles in the primary and metastatic tumors. (*see* Color Plate 1)

and Color Plate 1). However, predictive profiles are not significantly altered when comparing primary and metastatic lesions. As a result, the similarity of prediction profiles between primary and metastatic lesions argues against this hypothesis.

2.1. Cancer Stem Cell Theory of Metastasis

One explanation of metastasis that can account for the predictive ability of gene expression profiles invokes the stem cell model of cancer. This model holds that only a small population of cells within the tumor, called cancer stem cells or cancer-initiating cells, have the capacity to regenerate the tumor. These cells share a number of traits in common with normal tissue stem cells, including the presence of molecular pathways such as Notch, Hedgehog, and Wnt and high levels of ATP-binding cassette transporters, which actively efflux toxins from these cells, in order to protect their genetic material *(7)*. Because of these similarities, it is theorized that the origin of the cancer stem cell is the oncogenic transformation of a tissue stem or progenitor cell, generally found on site in adult tissues for regenerative purposes.

The idea that the tissue stem cell is the target for oncogenesis is attractive because hallmarks of cancer, self-perpetuation and the ability to travel, may already exist in this population. The number of mutations theoretically required to transform a tissue stem cell into a cancer stem cell—the small population of cells within a tumor that hold the capacity to self-renew and metastasize—should be less than the number of mutations it would take to transform a differentiated cell into a stem-like cell.

The predictive ability of a gene expression profile under this hypothesis can be explained by differences in the stem cell of origin between tumors. For instance, a stem cell, rather than a progenitor cell, as the oncogenic target would lead to a more aggressive and invasive tumor. No differences would be expected in the metastatic potential of primary tumors versus metastatic tumors, because the cancer stem cells in each subset are the same (Fig. 2b). Indeed, most of the cells at the metastatic site, just as with the primary site, have no metastatic potential at all, having been derived from the terminal differentiation of the cancer stem cell.

The migration program within normal embryonic stem cells is based on chemical cytokine gradients, which direct receptor-bearing cells to specific site destinations. One such receptor chemical cytokine system common in stem cells is the CXCR4 transmembrane receptor and its ligand, the chemical cytokine, stromal derived factor-1 (SDF-1). A role for the CXCR4 SDF-1 axis along with other cytokine/receptor pairs in metastasis has been described by Kaplan et al. *(8)* for the establishment of a premetastatic niche as a precursor to metastasis. Under the influence of tumor-secreted cytokines, such as vascular endothelial growth factor (VEGF) or placental growth factor (PIGF), fibronectin is deposited at a distant site. Further cytokine secretion then leads to the mobilization of hematopoietic progenitor cells from the bone marrow to the newly established fibronectin-rich areas in target organs. Only after this niche has been established, experimentally in the lungs of mice, do actual tumor cells begin to arrive under the influence of cytokine gradients established by the cells now at the metastatic site. Subsequently, endothelial progenitor cells, also derived from the bone marrow, arrive at the prepped site, completing the process of neovascularization and fully establishing metastasis. It has been shown in certain cancers that the tumor cells recruited to these organs are CXCR4 positive, though no definitive data are available to designate the mobile tumor cells as cancer stem cells. Nevertheless, this example of metastasis serves to illustrate the possible role of cancer stem cells in metastasis, as well as the role of those tumor cells that are unable to metastasize, but may still play an important role in the dissemination of tumor.

2.2. Germline Metastatic Potential

Another hypothesis on the origin of the metastatic potential seen in gene expression profiles expands considerably the number of genes that play a role in the determination of metastatic risk.

Using a polyoma middle-T antigen transgene model of mammary carcinoma in the mouse, Hunter et al. *(9)* showed that pulmonary metastasis was influenced by the genetic background of the animal: metastatic potential of mammary tumors was low in certain strains of mice, e.g., DBA and NZB, but high in others, e.g., FVB and AKR animals *(10)*. Relating this experiment back to the finding of predictive power in gene expression signatures, one must consider the orders of magnitude of differences in gene signatures based on inherited genetic background, versus the differences that would be generated based on mutations during development of a primary tumor. While tens to hundreds of genes contribute to the differences between primary and secondary tumors, that number is dwarfed by the number of genes that differ between individuals. A differential gene expression signature is apt to be dominated by these inborn genetic differences that lead to different metastatic outcomes, versus the relatively few acquired differences.

In this model, the conventional model of metastasis, wherein mutations are acquired late and metastases demonstrate higher metastatic potential than the primary tumor, remains viable. A gene expression signature would express not only the genetic difference in tumor, but the difference in the germline genetic background (Fig. 2c). Therefore, regardless of when mutations occurred in cancer cells, the signature would have its predictive power based on the genetic background that was favorable or not for metastasis. By the same token, even if only high metastatic potential cells comprised a metastasis, the genetic difference between it and a primary tumor would be negligible, compared to the background.

One of the genes that correlated with differential metastatic potential in Hunter's experiments, identified through haplotype mapping, is the signal-induced proliferation associated gene 1 (*Sipa1*) that encodes a GTPase-activating protein (GAP) for RAS family members Rap1 and Rap2 *(10)*. An alanine to threonine mutation in the PDZ domain of the molecule was present in mouse strains with high metastatic potential, as compared with those with low metastatic potential.

The relevance of *SIPA1* has been shown in human mammary cancer, as well. In breast cancer patients, haplotypes of *SIPA1* could be correlated with lymph node status and estrogen receptor status, both being prognostic factors for patient outcome *(11)*. Analysis of gene polymorphisms may therefore open the possibility to determine disease course not only from tumor tissue but rather based on the genetic background of the host/patient. While there are many ethical and privacy issues raised by such an eventuality, exploration down this path is a unique and promising approach to understanding and preventing metastasis.

3. TARGET GENES

Though the exact metastatic processes leading to differential gene expression signatures have yet to be established, investigators have been able to use the signatures as valuable tools in identifying the genes and pathways involved in promoting or preventing metastasis. Genes that are differentially expressed in both gene signatures described above are involved in the cell-cycle control, in angiogenesis, and in signal transduction. While many of the genes are expressed in the tumor cells, some were expressed exclusively in the stromal cells which emphasizes the role of the tumor stroma in tumorigenesis and metastases. A number of these genes are currently under investigation as targets in National Cancer Institute's (NCI's) intramural program, including transforming growth factor (TGF)-β and matrix metalloproteinase (MMP)-9.

TGF-β is a highly conserved, ubiquitous cytokine that is frequently elevated in human cancers. It is a prometastatic factor and, therefore, an attractive therapeutic target. However, it also inhibits growth of the primary tumor in early stage disease and modulates the function of the immune system *(12)*. To effectively use it in cancer therapy, it would be necessary to devise a

therapy that inhibits the prometastatic effect of TGF-β, while leaving intact its other immune and physiologic functions, including the ability to act as a tumor suppressor early in the neoplastic process.

Neutralization of TGF-β has been shown to reduce metastasis in two mouse models of breast cancer (*12*,*13*). Mouse mammary tumor virus (MMTV) driven expression of a TGF-β1,3 binding fusion protein, SR2F, which consists of the extracellular ligand-binding domain of TGF-β receptor II and the Fc domain of human IgG_1 in a *neu* based mouse model of breast cancer resulted in reduced metastases as compared with the control group that did not express SR2F *(12)*. Interestingly, expression of SR2F did not influence the number of primary tumors formed, tumor size, or latency, indicating that exposure to the antagonist does not inhibit TGF-β's tumor suppressor effect. Moreover, the advantage of SR2F therapy was conferred without any significant increase in major toxicities.

Similarly, neutralization of TGF-β in a metastatic orthotopic mouse model of breast cancer again showed that 4T1 inoculated animals that were treated with a control antibody had a twofold number of metastases, as compared with animals that received a TGF-β-neutralizing antibody *(13)*.

Given these promising results, investigators have turned their attention to developing a system to delivery the anti-TGF-β fusion molecule to human patients. Investigators believe that a feasible gene therapy approach can be developed by using the fusion protein to transfect an oncolytic adenovirus capable of replicating in all cancer cells, regardless of specific genetic defect *(14)*. Such a strategy could provide enormous benefits in halting or ameliorating the deadly progression to metastasis.

MMPs are known to aid in tumor progression and metastasis by breaking down the collagen IV in the extracellular matrix and basement membrane, and by releasing bioactive substances from the extracellular matrix *(15)*. MMP-9 expression levels are frequently altered in tumor cells and in the tumor stroma as well. In a 2D coculture system of dermal fibroblasts and human breast cancer cells (CA1a), but not in homotypic cultures, tumor cells stimulated fibroblasts to secrete MMP9 in a TGF-β, TNF-α, and EGF- and HGF-dependent fashion *(16)*. Interestingly, whereas tumor cell-derived TGF-β and TNF-α directly stimulated MMP-9 expression, HGF and EGF worked downstream of these factors, with HGF unable to induce MMP-9 expression on its own. In a model of colorectal metastases to the liver, MMP-9 knockout animals had a twofold decrease in liver metastases, as compared with wild-type animals. Again, MMP-9 expression in tumor tissue was localized in the tumor stroma *(17)*.

Though the discussion has focused on MMP-9 and TGF-β as targets crucial to halting the metastatic process, there are certainly numerous other genes implicated in the predictive profiles that have similar potential. Other examples include the metastasis suppressor, Nm23, that is downregulated in melanoma and, when overexpressed, reduces metastases in metastasizing breast cancer cell lines *(18)*; the Her 2 receptor tyrosine kinase, whose overexpression coincides with larger brain metastases in a mouse model of breast cancer although it does not influence the number of brain metastases *(19)*; and the cytoskeleton organizer ezrin that is overexpressed in rhabdomyosarcoma cell lines (*20–22*). Even genes identified on expression profiles that may not be amenable to therapeutic intervention can yield insights that can help to elucidate the pathways crucial to the metastatic process. Such insights would still be extremely valuable in designing therapy and prevention strategies.

4. FUTURE DIRECTIONS

Gene expression profiling has provided valuable targets of investigation in the tumor microenvironment and has challenged our previous understanding of the process of metastasis. Genetic profiling of both the tumor and the tumor microenvironment, as well as the stem cell

theory of cancer development and progression, has gained new prominence and has focused investigations on a small subset of cells within the tumor. When fully defined, these two areas of study may open up further avenues of inquiry. The definition and identification of cancer stem cells are avenues of investigation critical to uncovering the role of cancer stem cells in tumorigenesis and metastasis. Investigators strive to elucidate the role of genes, differentially expressed between patient groups with and without metastasis, whether these differences are in the germline or the result of somatic mutation. These and the myriad other strategies utilized at research institutions all over the world to meet the challenge of metastasis may prove critical to the development of preventive and therapeutic agents to halt the deadliest progression of cancer, thereby bringing us that much closer to the goal of eliminating the devastating effects of this disease.

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2 Vascular Endothelial Growth Factor: Basic Biology and Clinical Applications

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Contents

ABSTRACT

Vascular endothelial growth factor (VEGF)-A is a well-characterized angiogenic factor involved in physiological and pathological growth of blood vessels. The homologous tyrosine kinases VEGFR-1 and VEGFR-2 are the main VEGF-A receptors. Much evidence indicates that VEGF-A is important in tumor angiogenesis. Humanized anti-VEGF-A monoclonal antibodies and two small-molecule inhibitors of VEGF receptor signaling have been approved by the US Food and Drug Administration (FDA) for cancer therapy. Furthermore, VEGF-A is implicated in intraocular neovascularization associated with active proliferative retinopathies and the neovascular form of age-related macular degeneration (AMD).

Key Words: vascular endothelial growth factor; endothelium; angiogenesis; tyrosine kinases; tumor growth

1. INTRODUCTION

Angiogenesis is known to be fundamental to a variety of physiological processes including embryonic and postnatal development, reproductive functions, and wound healing *(1)*. Furthermore, neovascularization plays an important pathogenic role in tumorigenesis and in the vision

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loss associated with ischemic retinal disorders and the wet form of age-related macular degeneration (AMD). Research performed in recent decades has established that angiogenesis is a complex and coordinated process, which requires a series of signaling steps in endothelial and mural cells elicited by numerous families of ligands (reviewed in *[2]*). Moreover, a variety of endogenous inhibitors of angiogenesis have been identified, including endostatin, tumstatin, and vasostatin *(3)*. However, despite such complexity and potential redundancy, vascular endothelial growth factor (VEGF)-A appears to be necessary for growth of blood vessels in a variety of normal and pathological circumstances *(4)*. *VEGF-A* is the prototype member of a gene family that includes also placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and the orf-virus-encoded VEGF-E (reviewed in *[5,6]*).

Definitive clinical studies, resulting in approval by the US Food and Drug administration (FDA) of several drugs, have established that VEGF-A is an important therapeutic target for cancer and wet AMD (reviewed in *[7]*). This chapter summarizes the basic biology of VEGF-A and provides an update on the clinical progress in targeting VEGF.

2. HISTORY OF VEGF

Independent lines of research contributed to the discovery of VEGF *(4)*. In 1983, Senger et al. *(8)* reported the identification in the supernatat of a guinea pig tumor cell line of a permeability-enhancing protein, which was named "vascular permeability factor" (VPF). However, these efforts did not yield the full purification of the VPF protein. The lack of amino acid sequence information precluded cDNA cloning and elucidation of the identity of VPF. Therefore, very limited progress in understanding the role of VPF took place over the subsequent several years.

In 1989, we reported the isolation of an endothelial cell mitogen from the supernatant of bovine pituitary cells, which we named "vascular endothelial growth factor" (VEGF) *(9)*. The NH_2-terminal amino acid sequence of VEGF did not match any known protein in available databases *(9)*. Subsequently, Connolly's group at Monsanto Co. reported the isolation and sequencing of VPF *(10)*. By the end of 1989, we isolated cDNA clones encoding bovine $VEGF_{164}$ and three human VEGF isoforms: $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ *(11)*. The Monsanto group described a human VPF clone, which encoded a protein identical to $VEGF_{189}$ *(12)*. These studies indicater that, unexpectedly, a single molecule was responsible for both mitogenic and permeabiliy-enhancing activities.

3. BIOLOGICAL EFFECTS OF VEGF-A

VEGF-A stimulates the growth of vascular endothelial cells derived from arteries, veins, and lymphatics *(11,13)*. VEGF-A induces angiogenesis in a variety of in vivo models *(13)*. Administration of VEGF also induces rapid and transient increases in microvascular permeability in several experimental model systems (reviewed in *[14]*).

Inactivation of a single VEGF-A allele results in embryonic lethality between day 11 and day 12, indicating that during early development there is a critical VEGF-A gene-dosage requirement *(15)*. VEGF-A plays an important role also in early postnatal life. Administration of VEGF inhibitors, including monoclonal antibodies and soluble receptors, results in growth arrest and lethality in mice when the treatment is initiated at day 1 or day 8 postnatally *(16,17)*. VEGF is important for endochondral bone formation and growth plate angiogenesis and morphogenesis. VEGF-A blockade reversibly inhibits skeletal growth *(18)*. Another key function of VEGF-A is the regulation of the cyclical angiogenesis that occurs in the female reproductive tract *(19)*. VEGF-A is also a survival factor for endothelial cells, both in vitro and

in vivo *(20–23)*. VEGF induces expression of the anti-apoptotic proteins Bcl-2, A1 *(21)*, and survivin *(24)* in endothelial cells. In vivo, VEGF's pro-survival effects are developmentally regulated. VEGF inhibition results in apoptotic changes and regression of the vasculature of neonatal, but not adult mice *(16)*. Endothelial cells are the primary targets of VEGF-A, but several studies have reported mitogenic and non-mitogenic effects of VEGF-A on non-endothelial cell types including neurons (reviewed in *[25]*). It is now well established that VEGF-A promotes monocyte chemotaxis *(26,27)*.

4. VEGF-A ISOFORMS

Alternative exon splicing results in the generation of four main VEGF-A isoforms, which have respectively 121, 165, 189, and 206 amino acids after the signal sequence is cleaved ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) *(28,29)*. Less frequent splice variants have also been reported, including $VEGF_{145,}$ $VEGF_{183,}$ $VEGF_{162,}$ and $VEGF_{165b}$ (reviewed in *[13]*).

Like $VEGF_{165}$, native VEGF is a heparin-binding homodimeric glycoprotein of 45 kDa *(9, 30)*. In contrast, $VEGF_{121}$ lacks heparin-binding properties *(31)*. $VEGF_{189}$ and $VEGF_{206}$ bind to heparin with affinity comparable to that of bFGF *(31)*. Whereas $VEGF_{121}$ is a freely diffusible protein, $VEGF_{189}$ and $VEGF_{206}$ are almost completely bound to heparin-like moieties in the cell surface or in the extracellular matrix. $VEGF_{165}$ has intermediate properties in terms of heparin-affinity and bioavailability *(32)*. The long isoforms may be released in a diffusible form by proteolytic cleavage. Early studies showed that plasmin is able to cleave $VEGF_{165}$ at the COOH terminus, generating $VEGF_{110}$, a bioactive fragment consisting of the first 110 NH_2-terminal amino acids *(31,33)*. Interestingly, recent studies have shown that various matrix metalloproteinases (MMPs)—especially MMP-3- may also cleave $VEGF_{165}$ to generate diffusible, nonheparin-binding fragments *(34)*. Proteolytic processing of $VEGF_{165}$ by MMP-3 occurs in steps, with sequential cleavage at residues 135, 120, and finally at residue 113 *(34)*. Thus, the final product of MMP-3 processing, $VEGF_{113}$, is expected to be biologically and biochemically very similar to the plasmin-generated VEGF fragment.

5. VEGF RECEPTORS

VEGF-A binds two highly related receptor tyrosine kinases (RTK), VEGFR-1 *(35)*, and VEGFR-2 *(36)*. VEGFR-1 was the first RTK to be identified as a VEGF receptor more than a decade ago *(37)*, but the precise function of this molecule is still debated in the field *(13)*. The functions and signaling properties of VEGFR-1 appear to be varying with the developmental stage and the cell type, e.g., endothelial versus non-endothelial cells. VEGFR-1 binds not only VEGF-A but also PlGF and VEGF-B and fails to mediate a strong mitogenic signal in endothelial cells *(38,39)*. Non-mitogenic functions mediated by VEGFR-1 in the vascular endothelium include the release of growth factors *(40)* and the induction of MMP-9 *(41)*. Furthermore, VEGFR-1 mediates hematopoiesis *(42)* and monocyte chemotaxis *(27)* in response to VEGF-A or PlGF.

VEGFR-2 also binds VEGF-A with high affinity *(36,43)*. VEGF-C and VEGF-D may also bind and activate VEGFR-2, following their proteolytic cleavage *(6)*. The key role of VEGFR-2 in developmental angiogenesis and hematopoiesis is underscored by lack of vasculogenesis and failure to develop blood islands and organized blood vessels in Flk-1 null mice *(44)*. There is now agreement that VEGFR-2 is the major mediator of the angiogenic and permeability-enhancing effects of VEGF-A. VEGFR-2 undergoes dimerization and strong

ligand-dependent tyrosine phosphorylation in intact cells and results in a mitogenic, chemotactic, and pro-survival signal. Several tyrosine residues have been shown to be phosphorylated (for review see *[45]*).

In 1998, Soker et al. *(46)* identified a receptor for isoforms of VEGF-A containing the exon 7-encoded heparin-binding domain. Surprisingly, this receptor proved identical to Neuropilin-1 (NRP1), a molecule that had been previously shown to be implicated in axon guidance as a receptor for members of collapsin/semaphorin family *(46)*. NRP1 appears to present $VEGF_{165}$ to VEGFR-2 in a configuration that enhances the effectiveness of VEGFR-2-mediated signal transduction *(46)*.

6. ROLE OF VEGF-A IN TUMOR ANGIOGENESIS

Many tumor cell lines secrete VEGF-A in vitro (reviewed in *[13]*). In situ hybridization studies have demonstrated that the VEGF mRNA is expressed in many human tumors *(14)*. A variety of transforming events also lead to induction of VEGF gene expression. Oncogenic mutations or amplification of ras lead to VEGF up-regulation *(47)*. Renal cell carcinomas have a particularly high level of VEGF-A expression, consistent with the notion that inactivating mutation in the von Hippel–Lindau (VHL) tumor suppressor gene, resulting in high transcription of the hypoxia-inducible factor (HIF)-target genes under normoxic conditions, occur in ~50% of such tumors *(48)*.

In 1993, monoclonal antibodies targeting VEGF-A were reported to inhibit the growth of several tumor cell lines in nude mice *(49)*. Inhibition of tumor growth has been achieved also with other anti-VEGF-A treatments, including small-molecule inhibitors of VEGFR-2 signaling (reviewed in *[50]*), anti-VEGFR-2 antibodies *(51)*, and soluble VEGF receptors *(52,53)*.

Although tumor cells frequently represent the major source of VEGF-A, tumor-associated stroma is also an important site of VEGF production *(52)*. Recent studies have shown that tumor-derived PDGF-A may be especially important for the recruitment of an angiogenic stroma that produces VEGF-A and potentially other angiogenic factors *(54,55)*.

Combining anti-VEGF treatment with chemotherapy *(56)* or radiation therapy *(57)* results in a greater anti-tumor effect than either of these therapies alone. The mechanism of such potentiation is under debate. One hypothesis by Jain *(58)* is that antiangiogenic agents normalize the tumor vasculature. Alternatively, chemotherapy-induced damage to tumor endothelila cells may be amplified by blockade of a key pro-siurvival factor like VEGF *(56)*.

6.1. Clinical Trials in Cancer Patients with VEGF Inhibitors

Several VEGF inhibitors have been developed as anti-cancer agents. These include a humanized anti-VEGF-A monoclonal antibody (bevacizumab; Avastin™) *(59,60)*, an anti-VEGFR-2 antibody *(51)*, various small molecules inhibiting VEGFR-2 signal transduction *(50)*, and a VEGF receptor chimeric protein *(53)*. For recent reviews, see *(61–63)*.

The clinical trial that resulted in FDA approval of bevacizumab (February 2004) was a randomized, double-blind, phase III study in which bevacizumab was administered in combination with bolus-IFL (irinotecan, 5FU, leucovorin) chemotherapy as first-line therapy for previously untreated metastatic colorectal cancer *(64)*. Median survival and progression-free survival were increased by the addition of bevacizumab survival *(64)*. Although bevacizumab was generally well tolerated, some serious and unusual toxicities were observed. Hypertension requiring medical intervention with standard anti-hypertensive therapy developed in 11% of bevacizumab-treated patients. In addition, gastrointestinal perforation was noted in ~2% of

patients. In a combined analysis of five randomized trials involving bevacizumab, including the pivotal trial in colorectal cancer, the incidence of arterial thromboembolic complications, including stroke, myocardial infarction, transient ischemic attacks, and unstable angina was approximately double the incidence seen with chemotherapy alone.

The clinical benefit of bevacizumab is being evaluated in a broad variety of tumor types and lines of therapy. Several combination studies with biologicals are also ongoing, which include inhibitors of tyrosine kinase (bay 43-9006), the proteosome (bortezomib), and mTor (CCI-779). Bevacizumab combined with weekly paclitaxel in women with previously untreated metastatic breast cancer provided a substantial improvement in the primary endpoint of progression-free survival (11.0 versus 6.1 months, $p < 0.001$) relative to paclitaxel alone (reviewed in *[7]*). Combining bevacizumab with paclitaxel and carboplatin in patients with previously untreated, nonsquamous, NSCLC provided a significant improvement in the primary endpoint of overall survival (12.5 versus 10.2 months, $p = 0.007$) *(65)*. An earlier phase II study of bevacizumab in NSCLC had identified pulmonary bleeding as a significant adverse event in this tumor type *(66)*. Squamous cell histology was identified as a major risk factor for bleeding, and these patients were excluded from the phase III study. This markedly reduced the rate of serious bleeding associated with bevacizumab use *(65)*. Also, combining bevacizumab with 5-fluorouricil, leucovorin, and oxaliplatin (FOLFOX) in patients with previously treated metastatatic colorectal cancers provided a significant improvement in the primary endpoint of survival *(67)*.

Besides bevacizumab, several other types of VEGF inhibitors are being developed (reviewed in *[8,68,63]*). Among these, a variety of small-molecule RTK inhibitors targeting the VEGF receptors are at different stages of clinical development. The most advanced are SU11248 and Bay 43-9006. SU11248 inhibits tyrosine phosphorylation of VEGFRs, platelet-derived growth factor receptors (PDGFRs), c-kit, and Flt-3 *(69)* and has been reported to have efficacy in imatinib-resistant gastrointestinal stromal tumor (GIST) *(70)*. AG-013736, which has a similar spectrum of kinase inhibition as SU11248, has also shown therapeutic promise in metastatic renal cell carcinoma in a Phase II monotherapy study *(68)*. SU11248 is FDA-approved for the treatment of Gleevec-resistant GIST and metastatic renal cell carcinoma *(71)*. Phase III data indicate that Bay 43-9006 monotherapy results in a significant increase in progression-free survival in patients with advanced renal cell carcinoma *(72)*. In 2006, Bay 43-9006 and SU-11248 were approved by the FDA for the treatment of metastatic renal cell carcinoma.

7. ROLE OF VEGF-A IN INTRAOCULAR NEOVASCULAR SYNDROMES

The expression of VEGF-A mRNA is spatially and temporally correlated with neovascularization in several animal models of retinal ischemia *(20,73)*. This is consistent with the fact that VEGF-A gene expression is up-regulated by hypoxia, via HIF-dependent transcriptional activation *(74)*. In 1994, it was reported that the levels of VEGF-A are elevated in the aqueous and vitreous humor of human eyes with proliferative retinopathy secondary to diabetes and other conditions *(75,76)*. Subsequently, animal studies using various VEGF inhibitors, including soluble VEGF receptor chimeric proteins *(77)*, anti-VEGF-A monoclonal antibodies *(78)*, and small-molecule VEGF RTK inhibitors *(79)*, have directly demonstrated the role of VEGF as a mediator of ischemia-induced intraocular neovascularization.

AMD is the most common cause of severe, irreversible vision loss in the elderly *(80)*. AMD is classified as nonexudative (dry) or exudative (wet or neovascular) disease. Although the exudative form accounts for ~10–20% of cases, it is responsible for 80–90% of the visual loss associated with AMD *(81)*. Pharmacologic therapies for neovascular AMD have been approved by the FDA. One is verteporfin (Visudyne®) photodynamic therapy (PDT) *(82)* for only

predominantly classic lesions, in which 50% or more of the lesion consists of classic choroidal neovascularization (CNV). The other is Pegaptanib sodium (Macugen®) *(83)* approved in December 2004 for all angiographic subtypes of neovascular AMD. Although both treatments can slow the progression of vision loss, only a small percentage of treated patients experience any improvement in visual acuity. Most recently (June 2006), ranibizumab was approved by the FDA for the treatment of all subtypes of neovascular AMD *(84)*.

7.1. Clinical Studies of Anti-VEGF Therapy for Neovascular AMD: Pegaptanib and Ranibizumab

Pegaptanib sodium injection (Macugen®) and ranibizumab (Lucentis®) are the first ocular anti-VEGF treatments evaluated in large, randomized, controlled clinical trials for the treatment of neovascular AMD. Both are administered locally by intravitreal injection into the back of the eye. Pegaptanib sodium is a pegylated oligonucelotide aptamer that binds to and inactivates $VEGF_{165}$ *(85)*. In a combined analysis of the VISION trials— two identical, large, controlled, double-masked, randomized, multicenter clinical trials involving patients with all CNV lesion types—pegaptinib sodium prevented moderate vision loss (the primary endpoint, which was defined as loss <15 letters of vision) in 70% of subjects compared with 55% for the control group at one year ($p < 0.001$) *(83)*. However, on average, patients in the pegaptinib sodium group lost ~8 letters at one year, compared with a loss of ~15 letters in the sham injection group ($p < 0.002$). The proportion of subjects who experienced a moderate gain in vision (defined as a change of ≥15 letters at one year from baseline) was 6% in the pegaptinib sodium group versus 2% in the sham injection group ($p = 0.04$). Key adverse events observed in the pegaptinib sodium groups were uncommon and included endophthalmitis in 1.3%, traumatic lens injury in 0.7%, and retinal detachment in 0.6% of patients.

Ranibizumab (Lucentis®) is a recombinant, humanized Fab that binds to and potently neutralizes the biological activities of all known human VEGF-A isoforms, as well as the proteolytic cleavage product $VEGF_{110}$ *(84,86)*. Ranibizumab is currently being evaluated in two large, phase III, multicenter, randomized, double-masked, controlled pivotal trials in different neovascular AMD patient populations.

The MARINA trial randomized subjects with minimally classic (<50% of the lesion consisting of classic CNV) or occult without classic CNV to monthly sham injections or monthly intravitreal injections of one of two doses of ranibizumab *(87)*. In the primary analysis at one year, the study met its primary endpoint, with a significantly greater proportion of ranibizumab subjects avoiding moderate vision loss than sham-injected subjects. Moreover, on average, ranibizumab-treated subjects gained vision at one year compared with baseline while sham-injection subjects lost vision. A significantly larger percentage of subjects treated with ranibizumab gained ≥15 letters at one year than did the sham-injection group. Key serious ocular adverse events occurring in ranibizumab-treated subjects included uveitis and endophthalmitis and were uncommon.

The ANCHOR trial randomized subjects with predominantly classic CNV to verteporfin PDT with monthly sham ocular injections or to monthly intravitreal injections of one of two doses of ranibizumab with a sham PDT procedure. In the primary analysis at one year, the study met its primary endpoint, with a significantly greater proportion of ranibizumab subjects avoiding moderate vision loss compared with subjects treated with verteporfin PDT *(88)*. In addition, on average, ranibizumab-treated subjects gained vision at one year compared with baseline while verterporfin PDT subjects lost vision, and a significantly larger percentage of subjects treated with ranibizumab gained ≥15 letters at one year than did the verteporfin PDT group.

8. PERSPECTIVES

Research conducted for almost two decades has established that VEGF-A is important for regulation of the normal angiogenesis processes. Moreover, VEGF inhibition has been shown to suppress pathological angiogenesis in a variety of cancer models, leading to the clinical development of a variety of VEGF inhibitors. Definitive clinical studies have proved that VEGF inhibition, by means of bevacizumab in combination with chemotherapy, provides a significant clinical benefit, including increased survival, in patients with previously untreated metastatic colorectal cancer *(64)*. Furthermore, SU11248 and Bay 43-9006 have been recently approved by the FDA for metastatic renal cell carcinoma, and their mechanism of tumor suppression consists, at least partly, of inhibition of VEGF signaling *(61–63)*.

A particularly active area of research concerns the elucidation of the mechanisms of refractoriness or resistance to anti-VEGF therapy. Tumor cell-intrinsic or treatment-induced expression of angiogenic factors may be implicated *(89,90)*. Very recent studies have provided evidence that, at least in some murine models, refractorinessm to anti-VEGF therapy is related to the ability of the tumor to recruit CD11b + Gr1 + myeloid cells, which in turn promote angiogenesis *(91)*. It remains to be established whether these findings also apply to human tumors.

Most clinical studies with VEGF inhibitors have been conducted in patients with advanced malignancies. Preclinical studies suggested that such agents may be particularly effective when tumor burden is low *(92)*. Thus, the clinical benefit of these therapies might be greater if the treatment were initiated at earlier stages of malignancy. Adjuvant clinical trials with bevacizumab in breast, colorectal, and nonsmall-cell lung cancer patients are presently ongoing and the results should be known within the next few years.

Reliable markers are needed to monitor the activity of antiangiogenic drugs. Circulating endothelial cells and their progenitor subset are a potential candidate, as is MRI dynamic measurement of vascular permeability/flow in response to angiogenesis inhibitors, but neither has been clinically validated *(62)*. Emphasizing the difficulty of identifying predictive markers, a recent study found that VEGF-A and thrombospondin expression or microvessel density in tumor sections do not correlate with clinical response to bevacizumab in patients with metastatic colorectal cancer and patients showed a survival benefit from the treatment, irrespective of these parameters *(93)*.

VEGF inhibitors have demonstrated a marked clinical benefit also in wet AMD. Blockade of all VEGF-A isoforms and bioactive fragmemts with Ranibizumab not only slowed down vision loss, but unexpectedly appears to have the potential to enable many AMD patients to obtain a meaningful and sustained gain of vision. Further research is needed to determine whether the vision gain conferred by ranibizumab extends beyond 24 months and whether additional intraocular neovascular syndromes may benefit from this treatment.

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3 Proliferation and Cancer Metastasis from the Clinical Point of View

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CONTENTS

ABSTRACT

Lymph node metastases result from specifically lymphotrophic metastatic cells that may have no capacity to grow in vital organs, i.e., brain, liver, lung. Thus, lymph node metastases are predictors, not governors, of overall survival since they do not control survival. This is demonstrated by randomized trials, literature reviews, and meta-analyses of epithelial cancers which show no survival advantage comparing various types of nodal resection, or even observation only. Metastatic lymph nodes demonstrate the primary cancer's capacity to shed and disperse cells, but these shed cells display organ-specific spread. Thus, liver and lung resections for oligometastases cure patients because their metastatic cells may not involve other organs. Even extensive nodal metastases, by themselves, do not cause death, whereas extensive liver, lung, or brain metastases kill their host. Lymph node specificity is displayed in low-risk differentiated thyroid cancer, where 75% of patients have node metastases, yet have a 99% 20-year survival. Adjacent nodal metastases are removed in cancer surgery and become a prognostic marker, whereas other distant organs are not sampled as carefully, but also may harbor metastatic cells which could be prognostic markers if discovered. Genetic analysis of cancers predicts detailed biologic behavior that is more accurate than node metastases or size or grade, and will soon displace nodes as the most important prognostic marker, thus removing the necessity of removing lymph nodes.

Key Words: lymph nodes; lymph node metastases; breast cancer; axillary dissection; sentinel node biopsy

Over the past years, we have published a series of articles outlining our understanding of the role of lymphadenectomy in human cancers, particularly breast cancer *(1–13)*. These articles have explored the history of the knowledge and understanding of the lymphatic system, and the embryology, anatomy, physiology, immune function, and the role of the lymphatic system and

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lymph nodes in immune homeostasis generally, and its prognostic role in cancer patients in particular. This chapter will summarize briefly our assumptions based on a wide reading of the pertinent clinical and research literature, and clinical studies of our own.

There is great confusion and controversy currently about the role of lymph nodes and lymph node metastases *(3,11,12)*. While there are statistical relationships between lymph node metastases and survival outcome in most cancers, debates swirl about whether lymph node resections, with or without lymph node metastases, are related to survival, and about the meaning of micrometastases, or a few cells in the peripheral sinus of a sentinel node (called a submicrometastases), for instance *(3,11,12)*. There is the strange and biologically implausible suggestion that survival is increased by removing more, rather than fewer, negative lymph nodes in breast, gastric, and colorectal cancers, in the face of overwhelming evidence from randomized trials and other evidence-based data, that lymphatic resections of varying extent or even observation only do not confer or impair survival advantages *(3,12)*. Whether these retrospective reports about the alleged advantages of removing more negative nodes is the result of a "Will Rogers" effect from stage migration that occurs with more extensive tissue removal, or some other subtle patient selection process *(1)*, is unclear at present, but the possibility of an actual biological phenomena is highly unlikely, indeed improbable. Thus, the entire subject of lymph nodes, lymph node metastases, and the lymphatic system generally and their relationship to survival in cancer is hotly debated with a voluminous literature on various oncologic aspects *(3,11,12)*. There certainly is clear scientific data documenting that lymphatic cancer spread does not control survival outcome as metastases to the liver, lung, or brain do, while still retaining usefulness as a prognostic indicator *(3,12)*.

Lymphocytes and their collection in lymph nodes are cornerstones of the adaptive immune system—a flexible, alterable, malleable, late evolutionary development of a more sophisticated response to external "nonself," antigens (from viruses, bacteria, parasites, chemical toxins) *(14)*. The adaptive immune system supplements the innate immune system, a more evolutionarily primitive, fixed, nonadaptable, immunological function that is the first line of defense against external antigenic threats *(14)*.

There are only four known functions of the lymphatic system: (1) returning interstitial fluids to the circulation; (2) introducing absorbed nutrients from the gastrointestinal tract into the circulation—both these initial two tasks accomplished through the lymphatic vessels leading to the major lymphatic trunks that drain into the vascular system in the neck, or through other lymphatico-venus shunts; (3) exposure of antigens encountered at the body surfaces to lymph node lymphocytes by dendritic or other antigen-presenting cells (APC); and (4) the production of humoral antibodies and cytokine-mediated foreign antigen or cell-destroying substances produced by "B" and "T" lymphocytes of the adaptive immune system *(2,14)*.

The evolutionary development of the lymphatic system, from the earlier two features of fluid and nutrient capture, to the later immunological function as the principal site of antigen presentation and recognition and then homeostatic protection from foreign antigens via lymphocyte activity was critical to development of higher species and organisms *(11)*. Lymphocyte collections, initially scattered and near the body surface, were eventually also congregated into lymph nodes interposed in the lymphatic flow for production and distribution of defensive molecules that prevented destruction of the animal or organism from antigen products of external viral, bacteria, parasitic, or toxic chemical threats *(2,11)*. Blunting and abrogation of this lymphocyte-based defense system against foreign antigens is essential for allogenic organ transplantation, demonstrating its central role in immune homeostasis *(15)*. Transplantation lymphocyte paralysis is accomplished by various nonspecific chemotherapeutic agents and specific antilymphocyte globulins and molecules.

Some aberrations of the lymphocyte-based protection from foreign antigens are seen in autoimmune diseases that occur through complex breakdown of the ability to distinguish

"self" from "nonself" *(14)*. Many human cancers are apparently prevented from developing or kept dormant by immune surveillance mechanisms mediated through the lymphocyte-based adaptive immunological system as demonstrated by the frequency of some malignancies in immune suppressed transplant recipients, and in the rare immune incompetence diseases, particularly in children, but also in HIV/AIDS patients through destruction of some critical cell lines in the complicated adaptive immune defense.

Epithelial cancers are largely diseases of aging organisms, and the evolutionary development of the lymphatic-based adaptive immune system was blind to their emergence since, for the most part, cancers are more "self" than "other" and not a developmental concern of a system evolutionarily designed to deal with more immediately threatening common foreign antigens during reproductive age *(2,11,15)*. The fact that cancer cells can shed from their primary tumor and drift through the lymphatics to lymph nodes where they may lodge, die, grow, or pass through to the general circulation, is totally incidental to the usual homeostatic functions of the lymphatic immune system *(16)*. Additionally, since lymph nodes and regional lymphocyte collections are not vital organs, their involvement, and even regional destruction, in no way directly compromises survival of the organism *(11)*. Thus, at the level of ability to control survival, the regional lymphatics are unimportant, particularly in contrast to vital organs such as liver, lung, brain, and gastrointestinal tract, whose impairment leads to death. So even simple reasoning would indicate that an emphasis on removal or destruction of regional lymph nodes to improve survival is a misplaced concern about a biologically, or survival related, irrelevant organ system. This is emphasized by our reviews of randomized and nonrandomized trials of variations of regional lymphatic surgical removal which have thoroughly documented the disconnection of these lymphatic surgical procedures from overall survival *(3,12)*.

However, since surgeons largely deal only with the primary cancer and its immediately adjacent structures such as lymph nodes, they have endowed these components of surgical effort with the magical powers of survival control. This is akin to the saying "when all you have is a hammer (resection of primary cancer and lymph nodes), everything looks like a nail" (survival must result). Thus, when surgeons have in their armamentarium only primary tumor and adjacent lymph node removal, these aspects, in the case of adjacent lymph nodes, are endowed with unrealistic and unsubstantiated power—the power to control life. Primary cancer removal prevents further growth and dissemination of cancer cells, but adjacent lymphatic resection does not enhance that function. Destruction by removal of a regional lymph node basin harboring metastases cannot conceivably control the life of an organism in the same fashion as brain, liver, or lung metastases; yet, the surgical literature by and large fails to recognize these distinctively different implications and the differences between vital and nonvital sites of metastatic disease, and is still heavily focused on lymph node removal, whether involved with metastases or not. The greatest accomplishment of the concept and reliability of sentinel node biopsy is to at last achieve the goal of avoiding removal of regional and nodal lymphatic basins that do not harbor detectable metastatic disease, a decided break in the surgical traditional conception of lymph nodes as effective filters blocking dissemination of cancer cells *(10)*. It is important to acknowledge and accept the fact that lymph nodes are porous organs not filters, whose primary function is immunological *(17)*. If lymph nodes were effective filters, they would become blocked by normal shed cells, cellular debris, antigens and metastatic cells, and limb edema would be inevitable, a manifestation only rarely encountered naturally (elephantiasis from parasites) but not infrequently encountered subsequent to radical surgical nodal removal, particularly if accompanied by radiation therapy. The only life-endangering aspect of regional nodal metastatic involvement is in the unusual cases of extensive lymph node metastatic enlargement with subsequent blockage of adjacent hollow organs such as esophagus, bile duct, gastrointestinal tract, or ureters.

Another critical aspect to appreciate the interaction of the lymphatic system and metastatic cancer cells is the unique metastatic organ site specificity of shed cancer cells and clinical metastases arising from primary cancers *(11,16)*. This metastatic organ site specificity is seen clinically in liver-only metastases in colorectal cancers, islet cell tumors of the pancreas, carcinoid tumors of the intestinal tract, and ocular melanoma *(11)*. Lung-only metastases are seen with sarcomas and colorectal cancers, and lymph node-only-specific metastases are common in low-risk, young, thyroid cancer patients, and in carcinoid and pancreatic islet cell tumors. These last three examples of lymph node-specific metastatic pattern are remarkable for the lack of any relationship between the lymph node metastases, frequently multiple, and even recurrent nodal metastases, and survival *(11)*. Low-risk differentiated thyroid cancer patients have at least a 75% risk of nodal metastases when routine neck dissections are performed, yet have a 99% survival disease-free at 20 years *(18)*.

Resection of liver- and lung-isolated metastases that are few in number result in substantial long-term survival rates (20–40%), while resection of nodal metastases in the previous examples noted (thyroid, carcinoid, and islet cell cancers) results in nearly uniform survival. Thus, circulation of vast numbers of lymphatic-specific metastatic cancer cells over long periods of time fails to produce vital organ metastases in these unique manifestations organ-specific metastases. Likewise, liver or lung metastases resection resulting in long-term disease-free survival indicates that enormous numbers of circulating cancer cells that are liver or lung specific do not have the capacity to produce other organ site metastases.

This organ site-specific metastatic pattern has been amply demonstrated in animal research where separate genetic clones of experimental cancer systems clearly demonstrate organ-specific metastatic cells that when introduced systemically only lodge, grow, and produce clinical metastases in lung, or liver, or bone, or even more specifically the adrenal gland *(11)*. Such metastatic organ specificity mimics highly specific inflammatory reactions to foreign bacterial, viral, or parasitic encounters where antigen recognition and presenting cells coming from specific areas in the body alert lymph node lymphocytes and phagocytes that return to the original localized area of foreign antigen introduction to create local abscesses and local inflammation in attempts to eliminate these threats to homeostasis *(19)*.

Experimental animal systems exist where metastatic cells from human breast cancer lymph node metastases when reinjected systemically circulate back to, and reside and grow only in, lymph node stroma, convincing evidence of lymph node-specific metastatic cancer cell behavior *(20)*. This unique experimental lymph node-specific pattern could be abrogated by specific cytokines or other molecular manipulations, which again illustrates this unique lymph node organ specificity that leads to lymph node, but not other, metastases. This demonstrates why lymph node metastatic cells undoubtedly have no ability to grow in nonlymph node sites, just as resection of liver or lung metastases leading to long-term disease-free survival illustrates the highly specific nature of those innumerable circulating cells.

These clear examples of organ specificity of metastases illustrate that even though many millions of cancer cells circulate, lodgement and growth may be allowed only in specific organ sites by specific cellular features *(11)*. This demonstrates in another way why lymph node metastases and lymph node-specific cells pose no danger to vital organs; thus, liver-specific cells do not grow in lymph nodes and lymph node-specific cells do not grow in liver or lung. This also accounts for the lack of connection between lymph node metastasis removal and survival, since the nonvital organ lymph node metastatic cells do not increase the risk of vital, life-sustaining, other organ metastases.

The central theme, then, of lymphatic metastases is that of a prognostic indicator, but not governor of overall survival, of patients with cancer *(1,4,11)*. As cancers appear earlier in their disease course through screening, the proportion of patients with lymph node metastases

decreases substantially and among patients with nodal metastases those with more than three involved lymph nodes declines even more, reemphasizing that the need for regional nodal resections is restricted to that of regional control, which while of some importance, is a far lesser concern than survival.

Interestingly, even at very large sizes and with poor prognostic manifestations, the proportion of all cancer patients who display nodal metastases never exceeds about two thirds of patients, a further indication that lymph node metastases are never purely an expression of size or mechanical or prognostic features, but the result of complex interactions between lymphotrophic metastatic cells, host organ receptivity, and aspects of the organ microenvironment that fosters or prevents clinical metastatic involvement, particularly lymph node involvement. At least one third of advanced cancers apparently do not shed lymphotrophic cells, or shed cells that display lymphatic avoiding behavior.

Thus, lymph node metastases are not generating foci of vital organ metastatic clones (liver, lung, brain) and therefore, while of prognostic usefulness, their resection is not related to survival.

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4 Gastrointestinal Cancer and the Lymphatic System: Patterns of Micrometastasis and Lymphatic Mapping with Clinical Outcome

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Contents

ABSTRACT

Precise evaluation of lymph node status is the most important factor for determining clinical outcome in treating gastrointestinal (GI) cancer. Unfortunately, lymph node metastasis (MA) may not always be accurately assessed through a preoperative work-up. Because lymphatic drainage of the GI tract is much more complicated than other anatomical sites, skip MA occurs rather frequently. Currently, sentinel lymph node (SLN) mapping has become highly feasible and accurate for staging GI cancer, and has clearly developed into a new therapeutic modality.

The diagnosis of nodal MA, including micrometastasis (MM) using immunohistochemical and molecular technology, has been nearly achieved through GI cancer surgery. Furthermore, the sixth edition of the tumor node metastasis (TNM) classification has recently been redefined as "sentinel

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nodes (SN)," "micrometastasis (MM; 0.2–2 mm in diameter)," and "isolated tumor cells" (ITC; <0.2 mm). The clinical significance of MM or ITC in SNs may differ among various organs. In particular, the prognostic value of MM detected by molecular technology remains controversial.

The purpose of this symposium was to focus on the present status of SN navigation surgery in the three major types of GI cancers (esophagus, stomach, and colon), as well as to discuss the clinical significance of SLN biopsy, including the prognostic value of lymph node MA and MM identified in SN. The clinical impact of circulating tumor cells (CTCs) or bone marrow aspirates was also discussed.

All speakers showed favorable results for identifying the GI SLN. Thus, accurate detection and diagnosis of lymph node MA including MM in SLNs has achieved a selection of more sophisticated, tailor-made approaches in clinical practice.

Key Words: sentinel nodes; micrometastasis; isolated tumor cell; circulating tumor cell

Although more evidence from large-scale, multi-center clinical trials is required, SLN navigation may be very useful for individualized patient treatment. We believe that the SN concept will provide important therapeutic options, and its use may prove widely acceptable for GI cancer.

1. INTRODUCTION

The sentinel node (SN) concept and navigation surgery are breakthroughs in the surgical oncology of gastrointestinal (GI) cancer. Procedure of Sentinel Node Navigation Surgery (SNNS) has opened avenues for clinical practice in the field of GI cancer surgery, and individualized management approaches have become familiar *(1)*. In the treatment of GI cancer, precise evaluation of lymph node status is one of the most important factors determining patient clinical outcome. Recent development of SN mapping has clearly become highly feasible and accurate in the staging of GI cancer. Consequently, determination of lymph node metastasis (MA) status, including micrometastasis (MM), has become a crucial issue (Fig. 1 and Color Plate 2 This minisymposium focused on the current status of SN navigation surgery, identification of MM in SNs, and clinical outcome. Our primary interest is whether the SN concept is acceptable and can indicate the rational extent of lymph node dissection in GI cancer surgery, and whether the results of SN mapping reflect patient clinical outcome. Furthermore, which patients would benefit from adjuvant chemo-therapy? Individualized selective lymph node dissection for GI cancer based on SN status would seem to be a reasonable surgical approach. Upon application of SN navigation to GI cancer, there are three issues relating to oncology and lymphology. The first is the very complicated lymphatic stream from the GI tract, which varies in each GI organ, the esophagus, stomach, and colon. The second is the accuracy of MM in frozen examination during surgery, and the third is how to determine the appropriate extent of LN dissection in cases where SNs contain MA.

In this minisymposium, the clinical application of SN mapping and how it should be performed were clarified. Symposium participants learned that standard procedures are essential for further improvement of SNs' clinical application and standard methods for detecting MM in SNs. Whether the SN concept proves to be a useful tool for GI cancer chemo-radiation therapy was also discussed. This timely minisymposium brought together scientists and clinicians to clarify the central question of SN navigation for GI cancer. We hope that this symposium review will encourage readers to apply new approaches in their clinical practice.

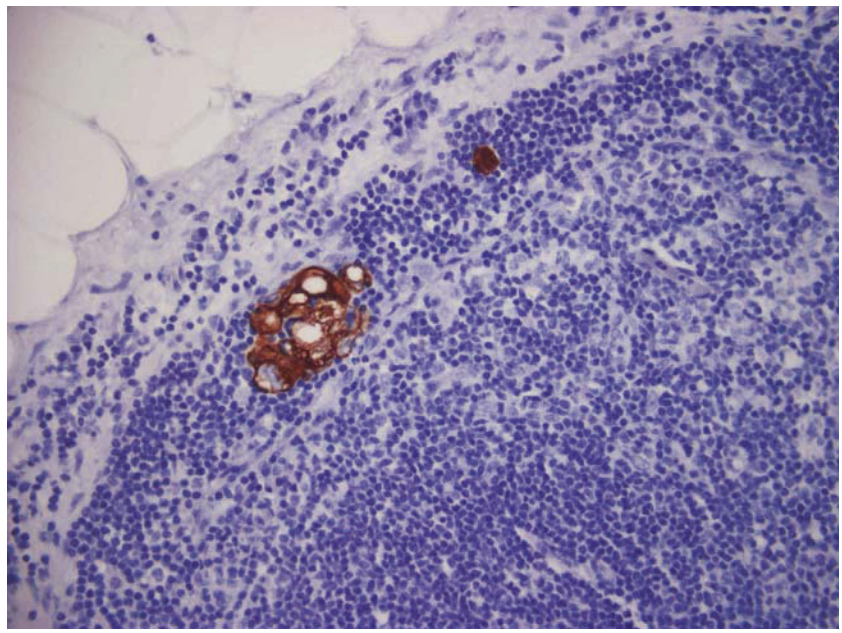

Fig. 1. Sentinel node with micrometastasis in gastric cancer. (*see* Color Plate 2)

2. CURRENT STATUS OF SN NAVIGATION SURGERY IN GI CANCER

2.1. Esophageal Cancer

Surgical resection of esophageal cancer is one of the most invasive treatments in the field of management of GI malignancies. Since the 1990s, the minimalization of surgical invasiveness to reduce postoperative complications has become a major topic in the management of relatively early GI cancers. In the field of esophageal surgery, initial challenges have been reported in 1993 by Cuschieri et al. *(2)* and Gossot et al. *(3)*. Although there are a number of controversial issues, technical and instrumental advances in thoracoscopic and laparoscopic approaches enable us to develop various minimally invasive surgeries for esophageal cancer. To secure the surgical curability of endoscopic esophagectomy, Kitagawa et al. *(4)* has been researching SNNS since 1999. In esophageal cancer, SNs are multiple and widely spread from cervical to abdominal areas. In more than 80% of cases, at least one SN is located in the second or third compartment of regional lymph nodes. This characteristic distribution of SNs is attributed to the multidirectional lymphatic drainage routes from the esophagus. A complete dissection of multiple and widespread SNs as nodes in a "functional" first compartment of regional lymph nodes is essential and oncologically important.

Kitagawa et al. are currently performing endoscopic surgery combined with SN navigation for T1 or T2 N0 esophageal cancer as a curative surgery. If intraoperative pathological and molecular diagnosis focused on harvested SNs shows the absence of MA, uniform extended lymph node dissection such as three-field lymphadenectomy is not necessary. Their idea is similar to our experience. Our colleagues, Uenosono et al., also studied 41 consecutive cT1 patients who underwent radical esophagectomy with regional lymph node dissection. The detection rate of hot nodes was 88% (36/41), and the accuracy of hot node status was 97% (35 of 36). Thus, radioguided SN detection is sufficiently reliable at present due to the prominent low-negative rate and high accuracy.

The possible existence of MM in superficial esophageal cancer cases is the most important factor in deciding therapeutic strategy. There are also limits to the diagnosis of the depth of tumor invasion by endoscopy and endoscopic ultrasound (EUS). In the near future, an individualized lymph node dissection and esophageal resection based on the distribution and status including MM of SNs will become feasible even in an endoscopic setting. Clinical application of SNNS will be expected to play a key role for individualized multimodal therapy in patients with cT1N0 esophageal cancer.

2.2. Gastric Cancer

Recently, the incidence of early gastric cancer has greatly increased; nearly 60% of patients in Japan show signs of early-stage gastric cancer. Thus, use of less invasive treatment such as an endoscopic mucosal or submucosal resection (EMR/ESD) has greatly increased. Minimally invasive surgery also has become the most common approach in early-stage gastric cancer. Many investigators in Japan have begun to apply the SN biopsy and navigation surgery by using dye and/or gamma probe-guided methods. However, there are central questions around the establishment of optimal procedure for SN mapping in gastric surgery, which have been the subject of much debate. One key question is whether dye or the radioisotope-guided (RI) method is more effective. Another important concern is determining the most suitable tracer and particle size, while other questions regarding the best timing or approach for tracer injection also remain.

The dye method is simple and easy, but there are some observation limitations due to the small size of the dye particles. Thus, a combination method, RI and dye, should be recommended due to anatomical locale and biological behavior of gastric cancer such as multidirectional lymphatic flow and skip MA. With regard to the RI method, ^{99m}Technetium(Tc)–Tin colloid is used, in which 3 mCi per 2 ml of Tc–Tin colloid is injected at four points surrounding the tumor using a gastro-endoscope 24 hours prior to operation. The most important point is the size of the Tin colloid. Of course, radioisotope uptake differs according to occupied tumor MA in SN. The importance of the suitable tracer size was reported in previous studies by Uenosono et al. *(5)*. The most suitable size of Tin colloid was 100 nm in GI cancer, because they are larger in the lymphatic vessels and clot in SNs. Another point of contention was the choice between submucosal injection and subserosal injection. Endoscopic submucosal injection might be superior because of the rich lymphatic vessel network in the stomach submucosal layers.

Up until recently, the results of sentinel mapping for early gastric cancer has been fruitful and favorable. Patients with clinically staged T1N0 gastric cancer are possible candidates for this procedure. The Aikou's group previously reported a sensitivity rate of 100%, a false-negative rate of 0%, and an excellent negative predictive value and accuracy in gastric cancer patients with cT1N0 tumor by H&E staining *(6)*. However, interestingly, we found one patient with false-negative results according to the immunohistological examination (Fig. 2).

Kitagawa et al. *(7)* also reported a detection rate of 95% and an accuracy rate of 98%. Thus, single-institution series have reported excellent results. Currently, to establish an optimal procedure of SN navigation surgery for gastric cancer, a prospective multicenter validation study for SN mapping in gastric cancer is being conducted by the Japanese Society of SNNS involving Japanese academic institutions.

SLN concept could be established in gastric cancer surgery from the viewpoint that quality of life was clearly improved by a reduction in lymph node dissections or the extent of gastric resection. SN navigation surgery in gastric cancer would be of great clinical significance if it were possible to perform sophisticated lymph node dissection.

	HE staining	IHC staining
Sensitivity	100% (13/13)	96% (26/27)
NPV	100% (134/134)	99% (122/123)
Accuracy	100% (147/147)	99% (146/147)

Fig. 2. Lymph node metastasis including micrometastasis in 147 Patients with cT1N0 gastric cancer.

2.3. Colon Cancer

Lymph node metastases are strong prognostic factors in colon cancer patients, as 20–30% of nodal-negative patients seen in current clinical practice will develop locoregional or systemic disease. An adequate lymphadenectomy during colectomy is thus an essential component of accurate staging. In the field of colorectal surgery, initial challenges have been reported in 2000 by Bilchik *(8)* and Saha *(9)*, who recommended a combination of radiotracer and blue dye method as the best technique for identifying SLNs. They emphasized that this technique will improve staging accuracy and reduce the morbidity of unnecessary lymph node dissections. Japanese surgeons expect that an SN biopsy for colon cancer will become an indicator for lateral lymph node dissection in cases of rectal cancer, as well as contribute to a more efficient diagnosis of MM.

There has been much debate as to which is better in colon surgery, the dye or RI method. Itabashi et al. *(10)* have been trying to detect colorectal cancer SNs using the dye method. Their findings showed that the skip MA frequency was 15.7% (50 cases) among 318 cases with lymph node metastases. The identification of SN was possible in 110 (98.2%) out of 112 cases. The diagnostic accuracy of lymph node MA with a dye method was 94.5%. Braat et al. *(11)* also reported similar successful SN identification in colon cancer using Patent Blue; their results are comparable with other SN studies using Lymphazurin. SN identification by dye method in colon cancers is a safe, fast, and easy procedure for ultrastaging the nodal basin. The technique involves a relatively flat learning curve and could become standard care for identifying the presence of nodal MM at a low cost, thereby also making it affordable at small healthcare centers.

In contrast, Covarelli et al. *(12)* presented preliminary results using radio-guided SN identification in colon cancer. The overall probe identification of SN was 95% (19/20), with one false-negative case where an MM in the SN was the only extracolonic site. This procedure also has the advantage that a gamma probe could be used to confirm radioactivity in the excised specimen with the absence of radioactivity in the surrounding operative field after resection.

Although SN mapping in colon cancer has enhanced staging accuracy, the utility of this technique for patients with colon cancer remains controversial. Bilchik et al. *(13)* published a series of 30 patients who had undergone laparoscopic SN mapping. They reported a 100% detection rate and a 93% accuracy rate, but the false-negative rate was relatively high (33%) due to the low number of patients with nodal disease (6 patients; 20%). Interestingly, in eight cases, aberrant lymphatic drainage was detected. The growing acceptance of laparoscopic colectomy for colorectal cancer has raised the question of whether SN mapping can be applied in laparoscopic procedure. Unless a standardized procedure is applied prospectively to these promising techniques, they will remain just that, promising.

3. IDENTIFICATION OF MICROMETASTASES IN SN

3.1. Esophageal Cancer

Esophageal cancer is one of the most lethal cancers, with a poor prognosis. Five-year survival for esophageal cancer is about 15%, even following surgical resection. This low survival rate implies the existence of MM, which is undetectable by histological examination. Recent publications have shown that undetected MA exists in negative lymph nodes, which relates to recurrence. The possible existence of MM in superficial esophageal cancer cases is the most important factor when deciding therapeutic strategy. Our colleagues, Natsugoe et al. *(14)*, analyzed initial site of solitary lymph node MA and MM in 65 consecutive esophageal cancer patients with solitary lymph node MA. The locations of MM were similar to those of solitary MA and paratracheal lymph node MA was associated with cervical lymph node MA in patients with thoracic esophageal cancer.

Tanabe et al. *(15)* clarified the prognostic value about immunohistochemically detected MM in lymph nodes from superficial esophageal squamous cell carcinoma (SCC). This study was conducted to determine incidence and clarify the patterns of nodal MM, to elucidate the histopathologic parameters of tumor extension correlating with MM, and to evaluate whether nodal MM has clinical significance in patients with superficial esophageal cancer. Lymph nodes resected from 78 patients with superficial esophageal SCC were examined immunohistochemically using the monoclonal antibody cocktail AE1/AE3 to define histologically undetectable MM. The authors concluded nodal MM is not rare in patients with superficial esophageal cancer, but does not appear to have clinical significance in these patients. Nodal MM correlates with intraesophageal multicentric cancer and venous invasion.

MM in SNs may differ in various organs. In particular, the prognostic value of MM in SNs detected by reverse transcriptase-polymerase chain reaction (RT-PCR) is still controversial. Kitagawa et al. *(16)* investigated the diagnostic and therapeutic significance of nodal molecular MA detected by nested RT-PCR for cytokeratin (CK) 19 mRNA in GI cancer. In 51 cases with GI tract cancer treated by standard curative resection, SNs were identified by a radioguided method. In 10 of 51 patients, 25 SNs and 3 non-SNs were histologically negative and RT-PCR positive. Three non-SNs with positive CK19 mRNA were randomly sampled from the same basin where histologically positive SNs were immunohistochemically analyzed during six additional step sections obtained at 30-μm intervals with an anti-CK antibody. Results showed clearly recognizable histological metastases in 4 of 25 histologically negative/RT-PCR-positive SNs (16%). In one case of esophageal SCC with nodal MM identified by CK19 RT-PCR, extranodal local recurrence in the SN basin (left supraclavicular basin) was observed six months postoperatively. These findings suggest that nodal MM detected by nested RT-PCR has some clinical significance in GI cancer. Molecular assessment of the SN may be a valuable tool to complement routine histological examination for GI cancers.

Immunohistochemical and experimental studies indicate that the concept of MM is applicable to esophageal cancer. New staging approaches, including immunohistochemistry and RT-PCR of various markers, seem to be useful for more accurate staging prior to esophageal surgery.

3.2. Gastric Cancer

Lymph node MA and MM are found in at least 20% of patients with early gastric cancer. Aikou et al. *(17)*reported that the incidence of MM in the lymph nodes was

considerably high, 24%, even if patients did not have lymph node MA based on routine histological examination. However, in patients who only had MM in the SN, lymph node MA was limited to the regional area near the primary tumor. Therefore, if lymph node MA cannot be detected by preoperative diagnostic means and immunohistological examination by frozen section in SNs during surgery, reduction of lymphadenectomy may be applicable to such patients using SN navigation.

If the utility of the SN concept is established in gastric cancer, it will become necessary to confirm pathological N0 or up-staging during the operation. In order to carry out clinical application of the SN concept, it is important to diagnose LN MA correctly. What is the accuracy of the intraoperative frozen section? How do we identify MM? Uenosono et al. clarified the value of the SN concept through identification LN MA and MM by H&E staining, immunohistochemical staining (IHC) and RT-PCR in 186 patients with a preoperative diagnosis of cT1 or cT2 gastric cancer. SNs were identified in 180 of 186 patients. The accuracy of SN navigation was 100% (146/147) in cT1N0 and 91% (30/33) in cT2N0. Only one false-negative case was found in cT1 patients by IHC.

Few reports have examined the morphological distribution of MM and ITC of SN in gastric cancer. Yanagita et al. *(18)* reported morphological distribution of metastatic foci in sentinel lymph nodes (SLNs) with gastric cancer and clarified the clinical significance of the morphological distribution of cancer cells in SNs according to MA, MM, and ITC. All dissected LNs obtained from 160 consecutive patients with mapped SNs arising from cT1-2 N0 tumors were examined. MA in these LNs was examined by histology and CK staining. The distribution of MA, MM, and ITC was classified as marginal sinus (MS), intermediate sinus (IS), parenchymal (PA), and diffuse types (DF) (Fig. 3 and Color Plate 3). Results can be summarized

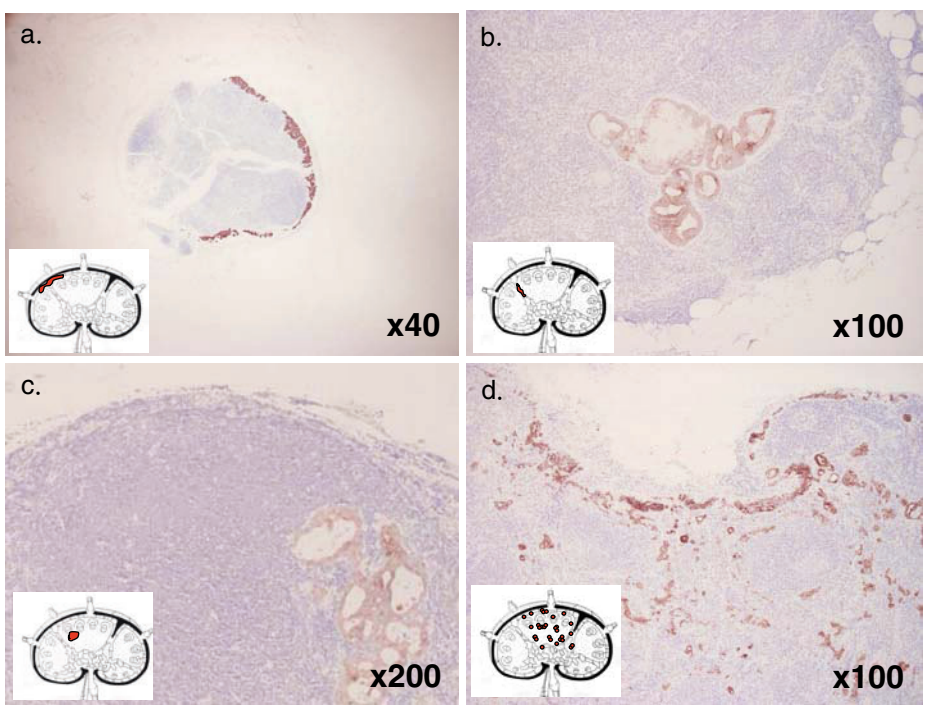

Fig. 3. Patterns of metastasis in sentinel nodes. **a.** Marginal sinus type, **b.** Intermediate sinus type, **c.** Parenchymal type, **d.** Diffuse type. (*see* Color Plate 3)

as: Nodal metastases were detected in 65 SNs from 30 patients and MA, MM, and ITC accounted for 53.9, 21.5, and 24.6%, respectively. MS, IS, PA, and DF accounted for 57, 6, 17, and 20.0%, respectively. When performing SN navigation surgery in gastric cancer, we should keep in mind the pattern of MM. Another issue is that immunohistological staining is labor-intensive and time consuming. We are currently developing a rapid detection method, GeneSearch™ intra-operative Assay (VERIDEX LLC., Warren, New Jersey, USA), which takes only 30 minutes to identify the gene indicative of MM in SN.

3.3. Colon Cancer

The 25% recurrence rate after complete resection of Stage II colon cancer suggests the presence of occult nodal metastases not identified by H&E staining. Bilchik et al. presented interim data from an ongoing prospective multicenter trial of SN biopsy, indicating a 24% rate of MM identified by IHC of H&E-negative SNs. SN navigation, however, does not apply to colon cancer where the morbidity of the operation is not dependent on the number of lymph nodes removed. Instead, the major issue in colon cancer is whether the resection specimen includes an adequate sample of lymph nodes. The principal role of SN sampling and ultra-staging in colon cancer is enhanced staging accuracy.

The newly published sixth edition of the *AJCC Cancer Staging Manual* makes a clear distinction between MM and isolated tumor cells (ITC), and it recommends guidelines for their reporting. Patients whose lymph nodes contain ITC (<0.2 mm in diameter) are classified as N0, with a modifier stating how the cells were detected. In the absence of prognostic data, patients with nodal MM (0.2–2 mm) are classified as N1. To comply with the new classification system, IHC will need to be performed on all lymph nodes or selectively on the SLN. Lymphatic mapping clearly is a better option because it accurately identifies MM and may help determine their prognostic significance

Clinical significance of MM detected by molecular techniques in SNs still remains controversy. A prospective randomized study comparing SN evaluation with standard pathologic evaluation for staging of colon cancer was conducted in patients with resectable colon cancer by the United States Military Cancer Institute Trial Group *(19)*. As a result, SN mapping, step sectioning and immunohistochemistry identified small volume nodal disease, showing an acceptable detection rate and sensitivity. Using lymphatic mapping, Paramo et al. *(20)* were able to detect micrometastatic disease in lymph nodes that stained negative for tumor by H&E. Their findings were similar to Bilchiks experience at the John Wayne Cancer Institute and of Saha et al. *(21)*. Other authors state that they upstaged 10–20% of the cohort using IHC. The universal application of this technique has been questioned because of inconsistencies in sensitivity and accuracy among a variety of authors *(22,23)*. However, it seems the SLN using serial step sectioning and immunohistochemistry (IHC) further enhance the ability to detect nodal tumor and accurately predict the status of the regional nodal basin. This procedure may contribute to the clinical application of the endoscopic surgical procedure, which has greatly increased in colorectal surgery.

Bilchik et al. demonstrated a high rate (up to 29%) of aberrant drainage in colon cancer patients undergoing lymphatic mapping at the most recent symposium. The 25% rate of recurrence among colon cancer patients with tumor-negative lymph node suggests inadequate staging, missed occult disease and/or aberrant lymphatic drainage pathways. All of these possibilities are addressed by lymphatic mapping and SN biopsy. SN mapping cannot only identify nodal MM, but can increase the number of tumor-draining lymph nodes identified by the pathologist. The results are more accurate staging and more appropriate selection of candidates for chemotherapy.

4. PROGNOSTIC IMPACT OF MICROMETASTASES

4.1. Esophageal Cancer

The concept of MM is a substantial surgical breakthrough. Immunohistochemical analysis (IHC), using epithelial markers, has been shown to identify micrometastases in histologically negative lymph nodes. The sixth edition of the TNM classification applied the concept of MM andITC. There has been much debate on whether single cells are viable, and whether the microinvolvement develops into true clinical MA. Natsugoe et al. *(24)* investigated frequency, associated tumor characteristics and impact on prognosis of MM and tumor cell microinvolvement (TCM) of lymph nodes from esophageal SCC. In this paper, MM was defined as individual tumor cells or tumor cell clusters <0.5 mm in greatest dimension with a surrounding stromal reaction. TCM was defined as individual tumor cells or tumor cell clusters without surrounding stromal reaction, and 1,954 lymph nodes were dissected from 69 complete resection specimens of TNM-classified pT1-3, pN0 or pN1, and M0 esophageal SCC. These lymph nodes were examined immunohistochemically using the monoclonal antibody cocktail AE1/AE3 for CKs. In conclusion, they suggested that, in SCC of the esophagus, MM, but not TCM, in the regional lymph nodes is prognostically equivalent to MA and should be examined by immunohistochemistry to correctly classify these cases as pN1. Matsumoto et al. *(25)* also reported clinical significance of lymph node MM of pN0 esophageal SCC. Lymph node MM was found in 39 (55.5%) patients, and tumor recurrence was found in 17 patients, all but one of which had MM. The five-year survival rate was significantly poorer in patients with MM than in those without MM.

Jiao et al. *(26)* recently performed a study to evaluate whether IHC analysis in thoracoscopic/laparoscopic (Ts/Ls) pretreatment staging lymph nodes can reveal additional diagnostic information to routine histopathology. Immunohistochemical analysis for CK can detect micrometastatic involvement of lymph nodes that are missed on routine pathologic examination and therefore can improve lymph node staging. Its clinical significance in esophageal cancer warrants further study.

A subgroup of patients considered N0 at standard single-section pathological examination may have occult micrometastases associated with a poor prognosis. Bonavina et al. *(27)* tested the hypothesis that 59 patients with esophageal adenocarcinoma undergoing resection were studied by standard histological examinations, serial sections, and immunohistochemistry. Their long-term prognoses were then compared. Eight (26%) out of 31 patients previously staged as pN0 at standard histological examination were staged as pN1 or pN2 by serial sections and/or immunohistochemistry and had a prognosis which was significantly worse than that of true pN0 patients (five-year survival: 38 vs. 76%, respectively; $p < 0.05$) and similar to that of pN1 patients. More than a quarter of those patients classified as pN0 at standard histological examination may have occult lymph node metastases at serial sections and/or immunohistochemistry and have a prognosis similar to that of pN1 patients.

4.2. Gastric Cancer

Recently, immunohistological staining using CK or genetic analysis for MM detection has become relatively easily available and familiar in gastric cancer. Although MM or ITC were defined in the sixth edition of TNM classification, measurement of these micrometastatic tumor cells is not easy. Some deposits were classifiable while classification of diffuse deposits was impossible. Other central questions necessary to clarify this include: Which has more impact on prognosis, volume of MM in the lymph node or number of micro-involved nodes? Which is more important on survival, character of micrometastasized cells or anatomical location of MM?

Furthermore, clinical significance of MM in gastric cancer has not been well clarified. Kim et al. *(28)* and other investigators reported that lymph node MM in histologically node-negative gastric cancer was significantly correlated with a poor five-year survival rate. The determination of E-cadherin expression in primary gastric tumor may be useful in predicting MM.

Interestingly, Yanagita et al. *(29)* clarified that SN micrometastases have high proliferative potential in gastric cancer. They examined the frequency and proliferative activity of such metastases, with a focus on the SNs of gastric cancer (Fig. 4 and Color Plate 4). Lymph node metastases in 133 patients with cT1-2 tumors (cT1: 104, cT2: 29) and mapped SNs were examined by routine histology and immunohistochemistry with anti-CK (Ki-67 antibody) to detect the primary tumor and lymph node metastases for evaluating proliferative activity. As a result, the number of patients with SN metastases and metastatic SNs was 19 and 52, respectively. The frequencies of macrometastasis, MM, and ITC were 48, 25, and 27%, respectively. Ki-67 expression in the tumor closely correlated with lymphatic invasion ($p = 0.0001$), venous invasion ($p < 0.0001$), and lymph node MA ($p < 0.0001$). Cells in 96% of macrometastases, 92% of MMs, and 29% of ITCs were Ki-67 positive (Fig. 5). These data showed that MM and some ITCs in SNs had proliferative activity. Thus, MM and ITCs should be removed, especially during SN navigation surgery, until their clinical significance is clarified.

Therefore our colleagues, Uenosono et al., performed clinical application of SN navigation for 61 patients with cT1N0 gastric cancer. In all patients, limited dissection of LNs was performed. MA by H&E was not found in all cases, but MMs were found in four patients by IHC and eight patients by RT-PCR. Although additional lymphadenectomy was not performed in all patients, they are currently alive without recurrence. RT-PCR with rapid

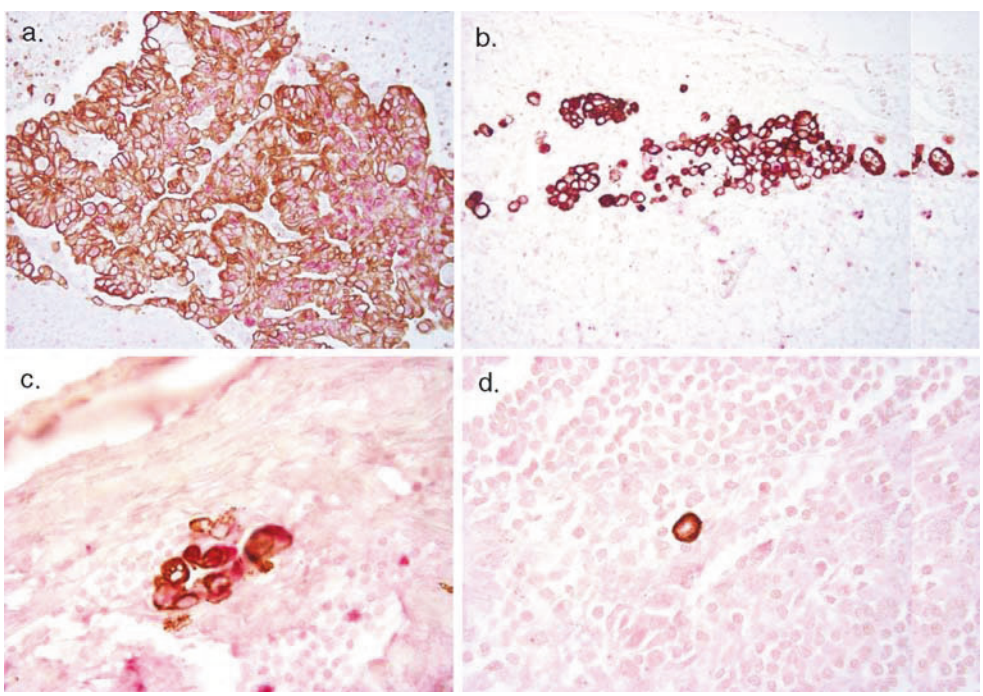

Fig. 4. Proliferative activity of metastatic foci. **a**. Metastasis, **b**. Micrometastasis, **c**. Isolated tumor cell, **d**. Single cell. (*see* Color Plate 4)

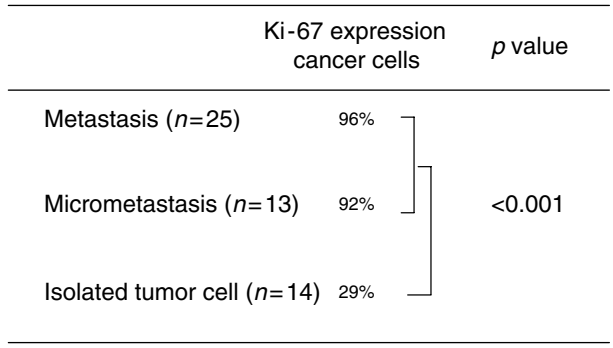

Fig. 5. Expression of Ki-67 in micrometastasis with gastric. 1. Micrometastases have high proliferative activity. 2. Some of Isolated tumor cells have proliferative activity.

protocols using GeneSearch^{TM} intra-operative Assay for gastric cancer has been developed. If the utility of GeneSearch^{TM} intra-operative Assay is confirmed, clinical application of SN navigation will be safer to perform.

What should be done about MM and ITC detected by molecular analysis, should they be treated or not? MM are currently designated pN1, but the relative risk of recurrence death in this population is uncertain. Although retrospective data indicate that patients with these nodes may be better served by an N1 classification, more evidence from large-scale multicenter clinical trials is required to confirm this finding.

4.3. Colon Cancer

The feasibility of SN mapping and its diagnostic reliability are now being determined in colorectal cancer. Recent reports published since 2000 suggest that they are promising, according to SN detection rates of 94–100% and overall diagnostic accuracy of regional lymph node status based on SN status of 86–100%. However, impact on recurrence and survival rates in colorectal cancer have not yet been verified between patients with and without MM in the SN. Although there is limited prospective data on the prognostic role of MM, new data indicate a similar prognosis for patients with nodal MM or node-positive disease. Wong et al. *(30)* suggest that the overall volume of metastases is not as important as the overall number of lymph nodes which are involved. It seems that all lymph nodes should be serial sectioned and stained with IHC and that there is no need for lymphatic mapping. Clearly, this is neither cost nor time effective. Wiese et al. *(31)* performed multilevel serial sectioned with IHC on all lymph nodes and found that nonsentinel MM were rare when the SLN was negative. This validates the power of lymphatic mapping for finding the node(s) that has the highest likelihood of harboring metastases.

Recently, Bilchik et al. *(32)* presented interim data from our ongoing prospective multicenter trial of SN biopsy. The incidence of MM identified by H&E, IHC, and/or quantitative RT-PCR was examined in 152 evaluable patients enrolled in the trial between March 2001 and August 2006. IHC and RT-PCR were performed on H&E-negative SNs. Results were correlated with disease-free survival. As a result, in a 25-month follow-up, 15 patients died from noncancer-related causes, 12 had cancer recurrence, 5 died of colon cancer (2 with macrometastases, 3 with MM), and 7 were alive with the disease. Of the 12 recurrences, 4 patients had SN macrometastases and 6 had SN MM (2 by IHC, 4 by RT-PCR). Of the 108 node-negative patients 23% had IHC MM and 2.8% had RT-PCR MM. The rate of recurrence was 2.6% (2 of 78) when SNs were negative by H&E or IHC/RT-PCR, compared with 16.4 (10 of 61) when SNs were positive by

H&E or IHC/RT-PCR ($p \leq 0.05$). One of two SN-negative recurrences, however, had other positive lymph nodes by H&E. This is the first prospective evaluation of the prognostic impact of MM in colon cancer. Results indicate that ultra-staging based on IHC/RT-PCR may improve selection of patients for adjuvant systemic chemotherapy, based on nodal evidence of MM.

As mentioned earlier, the prognostic significance of MM is a subject of intense debate. We agree that the presence of CK-positive cells within a lymph node at present has no known prognostic significance. We are even unsure whether or not they are truly tumor cells. Our laboratory uses differential tumor markers for evaluating whether these cells represent degenerating tumor cells, viable tumor cells, or benign mesothelial cells. Earlier studies using IHC to detect microscopic tumor in node-negative patients demonstrated no influence on overall survival; yet, studies using more advanced molecular techniques seem to suggest otherwise. A major problem is that until recently there has never been a standardized definition of MM.

5. CLINICAL SIGNIFICANCE OF CIRCULATING TUMOR CELLS AND BONE MARROW

5.1. New Paradigm of Microscopic Disease

The M.D. Anderson Cancer Center has formed the Advanced Research Center for Microscopic Disease, where the focus of research is related to the significance of minimal residual disease in patients with cancer. The group comprises medical oncologists, surgical oncologists, experts in stem cell transplantation, pathologists and immunologists, and currently performs translational and clinical research on aspects of minimal residual disease in breast cancer. Three types of minimal residual diseases of interest in breast cancer are the involvement of axillary SLNs, the presence of disease in the bone marrow, and the presence of circulating tumor cells (CTCs). At this meeting, Dr. Ueno discussed the research paradigm used for breast cancer, one that may be applicable to GI cancer.

The presence of CTCs in the peripheral blood in women with metastatic breast cancer, or that of CK-positive disease in the bone marrow in women with primary breast cancer, is considered an important independent prognostic factor. Change (reductions) in the numbers of CTCs after systemic treatment may predict the responsiveness of metastatic breast cancer before imaging studies show evidence of response. At the M. D. Anderson Cancer Center, they can routinely measure CTCs in women with metastatic breast cancer, as approved by the US Food and Drug Administration. Findings from a retrospective study have shown that having more than 5 CTCs at any time during metastatic disease is associated with worse progression-free or overall survival than having fewer than 5 CTCs. However, this finding must be confirmed by a prospective study involving large numbers of women with metastatic breast cancer. Moreover, the significance of CTCs and appropriate cutoff points in primary breast cancer are still being studied. The significance of different types of residual disease at each stage of the disease is being studied with the hope of using particular patterns of such disease to design individualized treatments.

Another important question is whether cancer associated with CTCs or minimal residual disease in the bone marrow is biologically different from other forms of primary or metastatic disease. Some studies have suggested that such cells express a "cancer phenotype" ($CD44^+$, $CD24^-$), suggesting that those cells may represent cancer stem cells. This observation has prompted intense genetic and epigenetic studies of these cells. In any event, the most clinically relevant question is whether modulating or targeting numbers of CTCs or cancer cells in the bone marrow can improve patient outcome. Thus, the real meaning of CTCs or bone marrow CK positivity in cancer, i.e., whether they represent a prognostic or predictive factor, stem cells or treatment targets, has yet to be defined. For this reason, a multidisciplinary team is needed to

answer these and other questions. Eventually, this research effort will be extended beyond breast cancer to pose similar questions on other forms of cancer. It is hoped that the answers will reveal common targets or mechanisms of minimal residual disease regardless of disease type.

5.2. CTC in GI Cancer

5.2.1. Esophageal Cancer

With respect to CTCs in perioperative esophageal cancer patients, quantitative assay system and potential clinical utility have remained controversial. Liu et al. *(33)* attempted to establish a quantitative system for evaluating the role of CTC in peripheral blood samples in patients undergoing esophageal cancer surgery. One hundred and fifty-five peripheral blood samples from 53 esophageal cancer patients were collected before surgery, immediately after surgery, and on the third day postoperatively. Eighty-nine samples were obtained from 22 benign patients who underwent thoracotomy and 30 healthy volunteers obtained as controls. A real-time RT-PCR quantitative analysis system based on carcinoembryonic antigen (CEA) mRNA gene expression was designed for detection of CTC. Their results indicated that there was a statistically significant difference between timing of collecting samples and esophageal cancer operation results in tumor cell dissemination and a significant increase of CTC in peripheral blood, which is related to the developed MA. CTC are helpful for evaluating MM and have the potential for predicting recurrence in esophageal cancer.

However, detection of MM to the bone marrow may predict widespread disease and a poor prognosis of cancer patients, thus requiring further elucidation. Nakamura et al. *(34)* evaluated the clinical significance of detecting MM in the bone marrow of esophageal cancer patients. Bone marrow and peripheral blood samples were obtained from 52 squamous esophageal cancer patients at the time of surgery. These samples were enriched by immunomagnetic separation and immunostained with an anti-CK antibody. Among them, three patients also had cancer cells in the peripheral blood. The presence of bone marrow MM was correlated with lymph node MA (pN), but not associated with depth of tumors (pT). Hematogenous recurrence was more frequent in patients with bone marrow MM than in those without. Natsugoe et al. *(35)* also evaluated the relationship between the presence of bone marrow MM, both before and after surgery, and clinicopathological findings in patients with esophageal cancer. Bone marrow samples from 48 patients with esophageal cancer were obtained from the iliac crest before and after surgery. In conclusion, recurrence and survival rates were poorer in patients with RT-PCR positivity, although these differences were not significant.

Detection of cancer cells in the bone marrow might be an indicator of early hematogenous MA in esophageal cancer patients. Intensive postoperative chemotherapy seems to be indicated for these patients. A larger study is therefore required to clarify the clinical impact of bone marrow MM.

6. CONCLUSION

The SN concept is a highly valued diagnostic tool in GI cancer surgery. This procedure offers real time results for surgeons; in turn, we are able to perform ultrastaging based surgery and offer individualized treatments. This symposium clarified the feasibility, diagnostic reliability and clinical application of SN navigation, and how it should be performed in GI cancer patients. This procedure may contribute to the clinical application of an endoscopic surgery procedure which has greatly increased in colorectal surgery. Each speaker demonstrated a novel and minimally invasive approach, such as SN navigation during endoscopic, laparoscopic, or laparoscopy-assisted surgery in patients with esophageal, gastric, and colorectal cancers. The available

data, however, do not justify ultrastaging by detecting MM at the molecular level or the choice of adequate treatment impact on prognosis. Another urgent task is to establish a standard method for detecting MM in the SN.

Although there are several remaining issues left for investigation, SN technologies will provide great tools in the establishment of individualized and minimally invasive surgical management of GI cancer.

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5 Redefined Lymphatic Anatomy of the Breast with Clinical Implications

Hiroo Suami, MD, PhD, Wei-Ren Pan, MD, and G. Ian Taylor, AO, MD

CONTENTS

ABSTRACT

Detailed gross anatomical information about the lymphatic system is essential for predicting accurate distant metastatic sites in solid cancers. Current anatomical knowledge of the lymphatic drainage of the breast is derived from studies by Sappey, Poirier, and Cuneo one century ago. The authors have developed a new radiographic protocol for delineating the lymphatic system in human cadavers; firstly using hydrogen peroxide to identify lymphatic vessels in the soft tissues, then injecting a radioopaque lead oxide mixture directly into these vessels and recording their findings on radiographs. We found that the lymph vessel drainage patterns of the breast and upper torso showed no significant difference between male and female specimens. Some lymph vessels that originated at or below the costal margin of the chest, coursed through the breast tissue to reach the axilla. Other lymph vessels coursed beside the perforating branches of the internal mammary blood vessels to reach the internal mammary lymphatics. These findings are discordant with our basic knowledge of breast lymphatic drainage, thereby initiating our anatomical review of the lymphatics of this region.

Key Words: sentinel node biopsy; cadaver study; breast lymphatics; lymph-collecting vessel

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1. INTRODUCTION

Since Virchow reported the importance of swelling of the left subclavicular lymph node as an indicator of metastasis of an early gastric cancer in the mid-nineteenth century *(1)*, cancer researchers have been investigating lymphogenic cancer spread from primary solid tumor to distant metastasis. Halsted *(2)* proposed that the axillary clearance operation and en bloc resection were the best way to control breast cancer metastasis before cancer cells could enter the blood stream via the lymphatic system. His rationale has been the dogma for breast surgeons for a century. However, a recent multicenter randomized clinical study, the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-04 trial, revealed axillary clearance does not contribute to the survival of breast cancer patients *(3,4)*.

The lymphatic network is concentrated in the skin, gastrointestinal tract, and airways and provides information to the immune system to protect the human body from external pathogens and internal neoplasms. The lymphatic pathway originates as a lattice of avalvular lymph capillaries which drain to valved precollectors in the dermis and then to lymph-collecting vessels (lymph collectors) in the subcutaneous tissue (Fig. 1). The lymphatic system is classified into the superficial and deep system separated anatomically by the deep fascia *(5)*. The lymph-collecting vessels have a smooth muscle layer that provides peristalsis to convey lymph fluid centrally to their regional lymph nodes *(6)*. These vessels are regarded as the main pathways of the lymphogenic cancer metastases.

Lymph fluid is almost colorless and the vessel walls of lymph-collecting vessels are very thin. It is almost impossible with the naked eye to distinguish the lymph vessels clearly from the surrounding

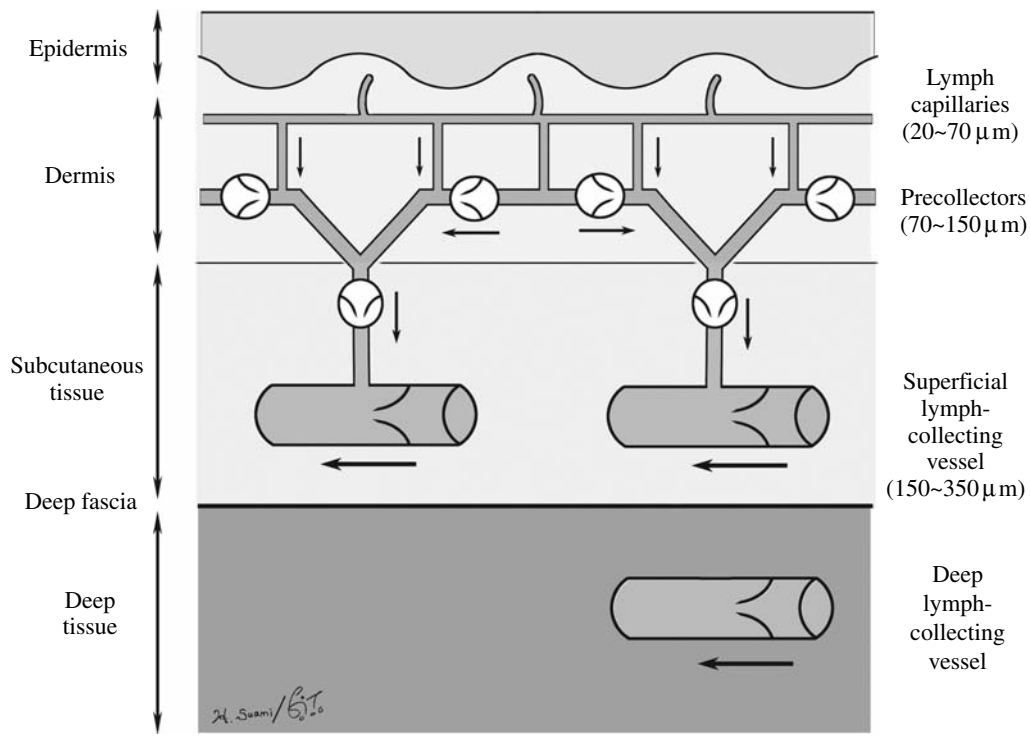

Fig. 1. Schematic diagram of the lymphatic system. The lymphatics start as capillary and precollector network in the dermis and then drain to subcutaneous lymph-collecting vessels. (From Suami H et al. The lymphatic anatomy of the breast and its implications for sentinel lymph node biopsy: a human cadaver study. Ann Surg Oncol 2008; 15:883–871. Reprinted with permission).

tissue. It is essential therefore to stain lymph vessels for gross anatomical studies. From the eighteenth century to the early twentieth century anatomists used mercury for this purpose and compiled excellent atlases *(7–9)*. However, their drawings are believed to be composite pictures from multiple specimens and therefore do not accurately represent current sentinel node concepts.

We developed a radiological injection technique for demonstrating the lymph-collecting vessels in a cadaver *(10,11)*. This technique enabled us to not only trace the longitudinal pathways of lymph vessels from their origin to their corresponding first echelon node (sentinel node), but allowed us to perform cross-sectional views of the female breast, thus demonstrating the 3D spacial relationship of the lymph vessels in the breast tissue.

2. HISTORICAL REVIEW OF THE BREAST LYMPHATICS

There are very few reports of the gross anatomy of the breast lymphatic drainage despite its clinical significance for predicting cancer metastases. Cruickshank is probably the first person to describe the anatomy of the breast lymphatics in detail *(12)*. He used young lactating female cadavers for his studies and by chance observed breast lymphatics when he injected mercury retrogradely into the mammary duct from the nipple. In 1840, Cooper, who is well-known for Cooper's ligaments of the breast, injected mercury into a cadaver breast and delineated lymph vessels from the nipple to the apical node (Fig. 2) *(13)*.

Sappey is credited with our current anatomical knowledge of the lymphatic system. He also used mercury and performed an extensive and comprehensive study of each region of the body using human cadavers *(8)*. He described the breast lymphatics originating in the vicinity of the mammary lobules and draining firstly into the subareolar lymph plexus. He stated that they usually united into two large lymph-collecting vessels in the areolar region which then drained directly toward the axilla. His findings became the background rationale for the current subareolar injection technique for sentinel node biopsy *(14,15)*. His diagrams of the male anterior upper torso and female breast were depicted in separate pictures (Figs. 3 and 4). In 1903, Poirier and

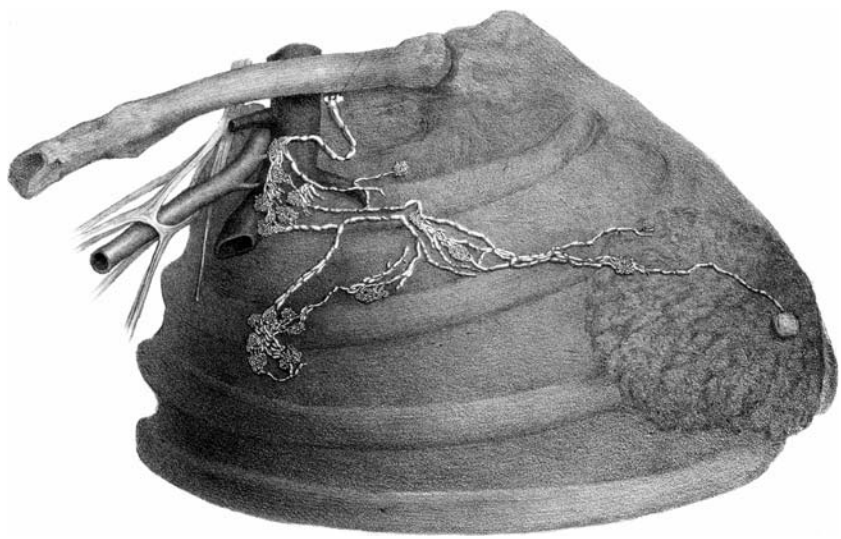

Fig. 2. Cooper's mercurial injection of the breast lymphatics from the nipple to the apical lymph nodes. (From Cooper *[13]*).

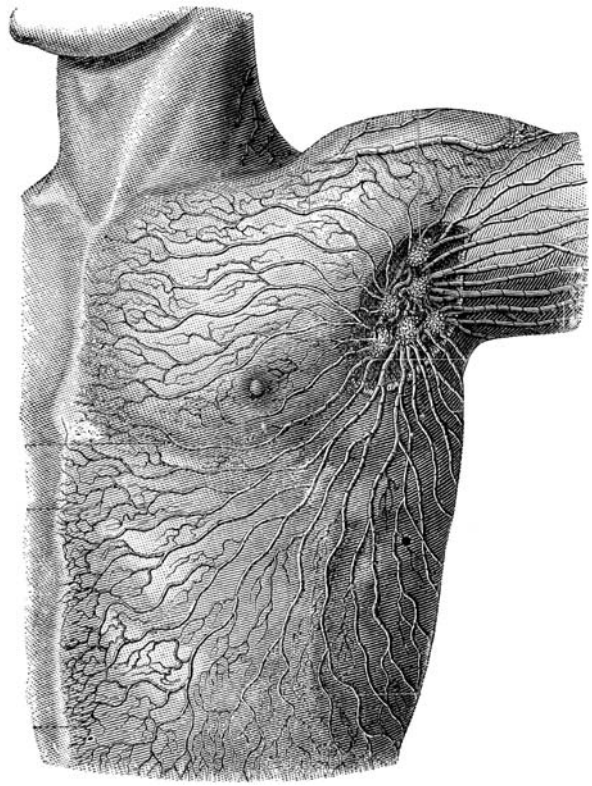

Fig. 3. Sappey's mercurial injection of the anterior upper torso. (From Sappey (1874) *[8]*).

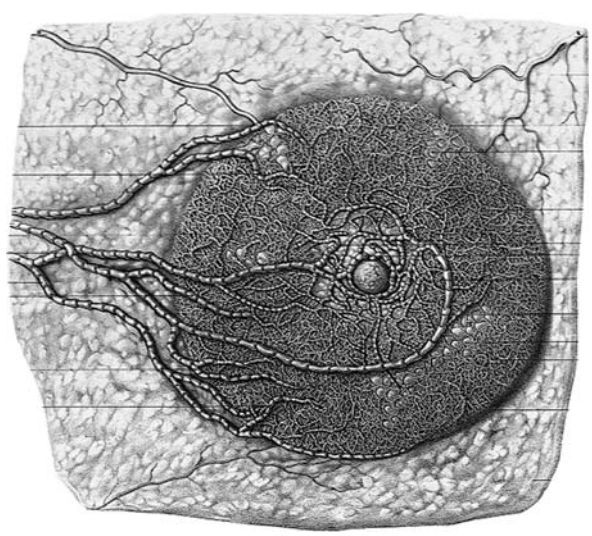

Fig. 4. Sappey's diagrams of the breast lymphatics of pregnant woman. (From Sappey (1874) *[8]*).

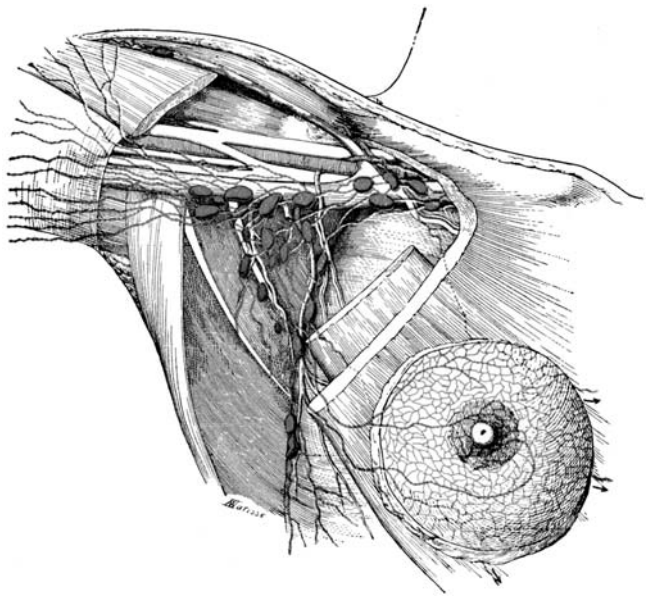

Fig. 5. Poirier and Cuneo's review diagram of the breast lymph drainage. (From Delamere et al. (1903) *[9]*).

Cuneo *(9)*, disciples of Sappey, published a diagram of the breast lymphatic drainage in their book (Fig. 5). The diagram was a composition of the anatomical and clinical findings of several studies including Sappey's. This diagram was redrawn and is still being used in Gray's Anatomy*(5)*.

3. MATERIALS AND METHODS

Fresh unembalmed human cadavers of medium size with no scars and noncancer death were selected. Bilateral anterior torso specimens that included clavicle, sternum, and ribs, were harvested with an incision across the root of the neck, down the posterior axillary line, and across the abdominal wall, above the umbilicus.

Firstly, a cannula was inserted into each internal mammary artery at its proximal cut end and a mixture of Indocyanine Green dye and gelatin was injected to stain the internal mammary artery and its nutrient branches to the breast. Six percent hydrogen peroxide was used to identify lymphatic vessels. When hydrogen peroxide is injected into the dermis and subcutaneous tissue around the targeted area, it reacts vigorously with the tissue enzymes and produces fine oxygen bubbles. Inflated lymphatic vessels can be identified under the surgical microscope (Stemi 2000, Carl Zeiss Pty. Ltd., Germany).

Small veins are also distended by oxygen bubbles, but there are two simple distinguishable differences: firstly veins contain red blood cells and secondly lymph vessels take on a bead-like appearance because of a thinner vessel wall and numerous valves. After identifying the lymph vessels, radio-opaque lead oxide mixture (orange) was injected into the lymphatic vessels. Dissection and radiographic examination then followed. A micromanipulator (MN-153, Narishige Co., Japan) (Fig. 6) was used to steady the 3D cannulation of a fine glass tube and 30 G 1 inch needle (Precision glide needle, Becton Dickinson & Co., USA). If a lymph vessel was not filled completely with a single injection, the cannulation and injection process was repeated until the injectant reached the first echelon node. This procedure was used along the entire

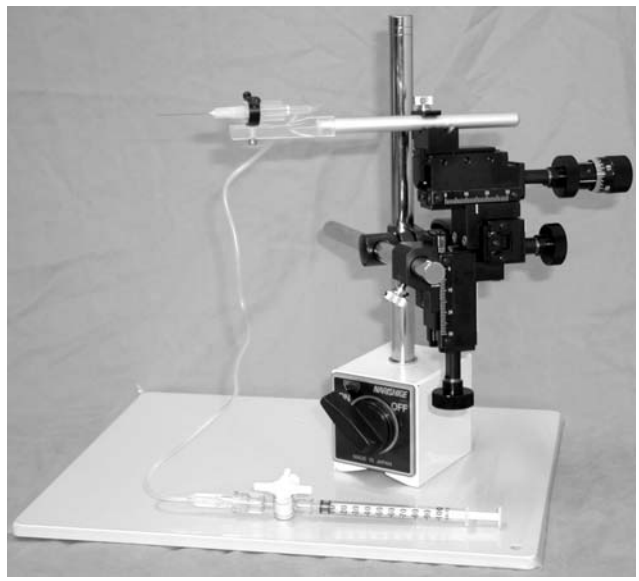

Fig. 6. A micromanipulator for cannulating a glass tube and a 30 G 1 in. needle. (Narishige Co.).

lymphatic system, from the bottom cut end of the abdominal wall, the midline, the areolar region, and in the vicinity of the branches of the internal mammary arteries that supply the breast. After completing the injection of all the lymph vessels found, the specimens were radiographed to demonstrate 2D views of the breast and anterior upper torso lymphatics (Fig. 7). Each lymph

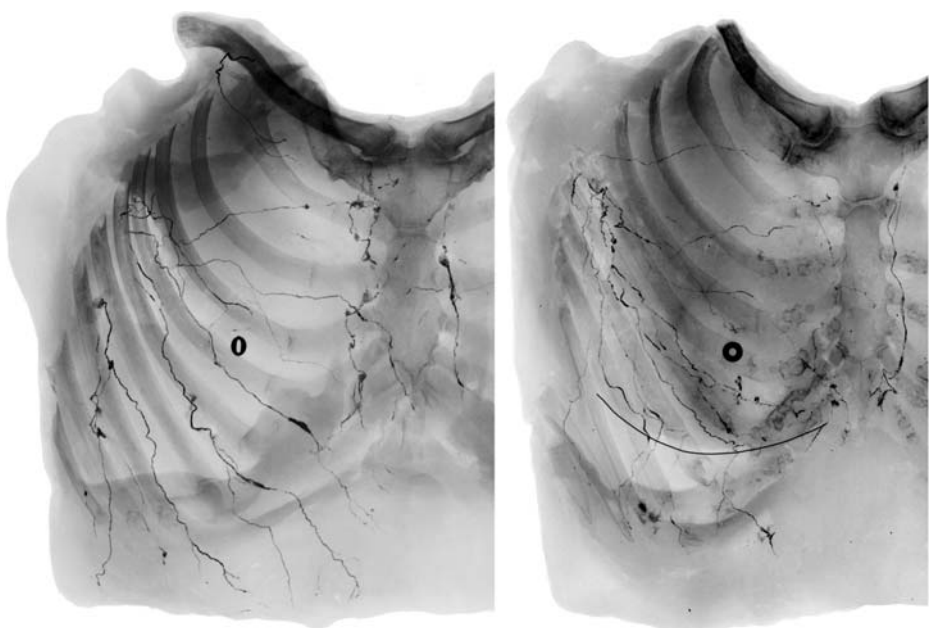

Fig. 7. Antero-posterior radiographs of a male (*left*) and female (*right*) specimen after completing injections with the lead oxide mixture. (From Suami H et al. The lymphatic anatomy of the breast and its implications for sentinel lymph node biopsy: a human cadaver study. Ann Surg Oncol 2008; 15:883–871. Reprinted with permission).

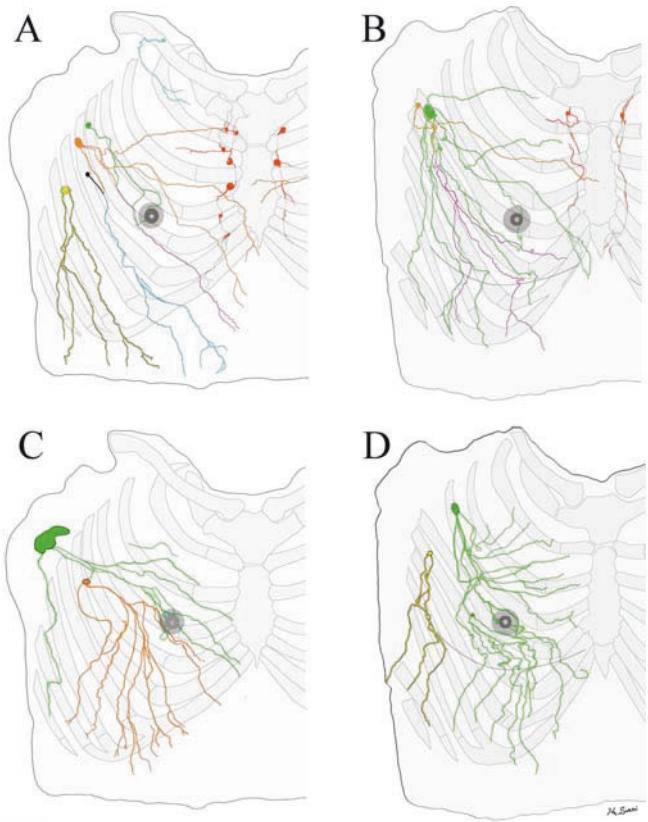

Fig. 8. Tracing of lymphatics of both hemi upper torsos (Male: **A** and **C**, Female: **B** and **D**) retrogradely from each first echelon lymph node colour coded. NOTE: (i) lymph-collecting vessels from nipple and areolar region on each specimen drain into the *green coloured* lymph node; (ii) the similar pattern of chest and breast drainage between the male and female studies; (iii) the breast lies in the pathway of collecting lymphatics that start peripherally; and (iv) that although the breast lymphatics drains to one sentinel node in **D**, every breast area is drained by more than one first tier node in each study. (From Suami H et al. The lymphatic anatomy of the breast and its implications for sentinel lymph node biopsy: a human cadaver study. Ann Surg Oncol 2008; 15:883–871. Reprinted with permission). (*see* Color Plate 5)

vessel was traced retrogradely from its corresponding sentinel node and color coded (Fig. 8 and Color Plate 5). Using the female breast, three incisions were made radially from the axilla in a fan-like fashion. Each "wedge" contained lymph vessels that had been traced from the subcostal margin. These specimens were placed on their side and were radiographed in order to investigate the 3D spacial relationship between lymph vessels, skin, and breast tissue (Fig. 9).

4. SUPERFICIAL LYMPHATIC SYSTEM

The superficial lymphatic vessels were found at or below the subcostal margin, the lateral border of the sternum, and in the areolar region. They radiated centripetally toward the axillary Level 1 lymph nodes. The lymph vessels were found at regular intervals in the peripheral region. In contrast to the veins, the lymph vessels retain a uniform diameter until they reach the sentinel lymph node. The arrangement of lymph vessels of the breast and anterior upper torso is similar in both sexes (Figs. 7 and 8). The breast lymphatic system is not an independent functional unit but a part of the lymphatics of the anterior upper torso which pass over or through its substance en route to the axilla.

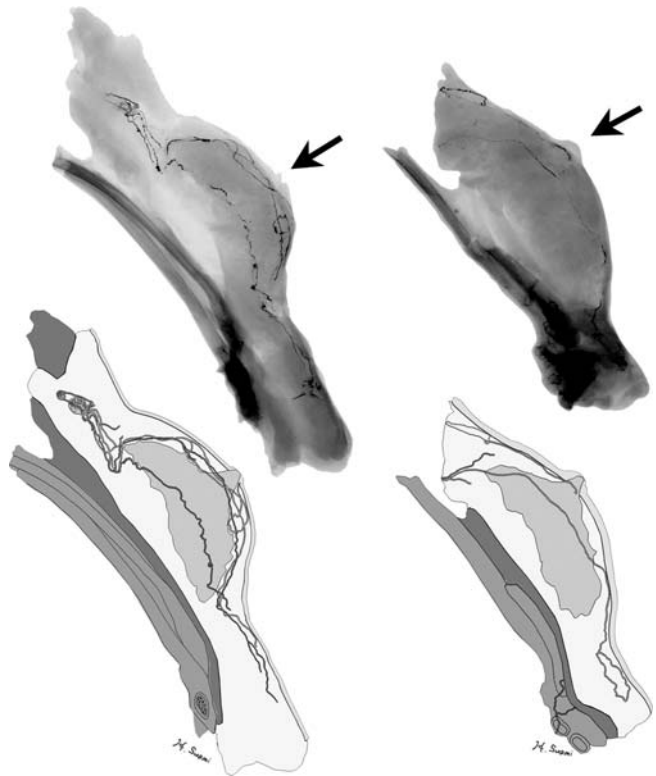

Fig. 9. Radiographs (*above*) and diagrams (*below*) of the cross-sectional views of the female breast including the nipple (*arrow*). (From Suami H et al. The lymphatic anatomy of the breast and its implications for sentinel lymph node biopsy: a human cadaver study. Ann Surg Oncol 2008; 15:883–871. Reprinted with permission).

This method enabled us to identify the sentinel lymph nodes that drain the breast. The numbers were variable; in some specimens, one sentinel node in the axilla drained the entire breast, and in other specimens there were multiple sentinel nodes involved (Fig. 8).

Cross-sectional radiographs of the female breast demonstrated the 3D spacial relationship between the lymph vessels and the breast tissue. Most of the lymph vessels originating from the lower part of the anterior upper torso ran between the skin and the breast tissue; however, some passed through the breast parenchyma (Fig. 9). Some lymph vessels originating from the areolar region merged with a torso vessel before reaching the sentinel node, others did not.

The authors could not prove conclusively that the lymph vessels passing through the breast drain the breast lymphatics. However, it does conflict with early studies that all the breast lymphatics collect centripetally in the subareolar plexus. Turner-Warwick mentioned that lymphatic pathways from the breast tissue drained into the axilla without passing through the subareolar plexus. He stated that nipple areolar staining by a peripheral intramammary injection of dye was caused by dye entering the lactiferous system instead of the lymphatic system *(16)*. Recent lymphoscintigraphy examinations also show these direct pathways *(17)*. Therefore, the authors have come to the conclusion that the lymph vessels that pass through the breast contribute to breast lymph drainage and are the same vessels demonstrated in lymphoscintigraphy.

5. PERFORATING LYMPHATIC SYSTEM

As well as the axillary lymph nodes, the internal mammary lymph nodes are the primary lymphatic basin of the breast. Turner-Warwick's radio tracer studies reported that every breast quadrant drained into both the axillary and the internal mammary nodes *(16)*. Cruickshank noted the existence of this pathway in his cadaveric studies 200 years before *(12)*. However, detailed pathways from the breast to the internal mammary node have never been recorded radiographically.

The principal nutrient branch from the internal mammary blood artery to the breast passes through the second or third intercostal space *(18)*. The perforating lymph vessels were found around these blood vessels (Fig. 10). These lymphatic vessels coursed deeply beside the blood vessels, passing through the intercostal muscles and deep fascia to reach the internal mammary lymph nodes. The blood supply to the breast showed individual variability *(19)*.

Radioopaque mixture injected into the superficial lymph-collecting vessels never flowed into the perforating lymph vessels. There was no distinct connection between the superficial lymph-collecting vessels and the perforating collecting lymph vessels. Current anatomical classification of the lymphatic system is divided into the superficial and deep lymphatic system with reference to the deep fascia *(5)*. We have subdivided the superficial lymphatic system into the conventional horizontal superficial lymphatic system and the vertical perforating lymphatic system (Fig. 11).

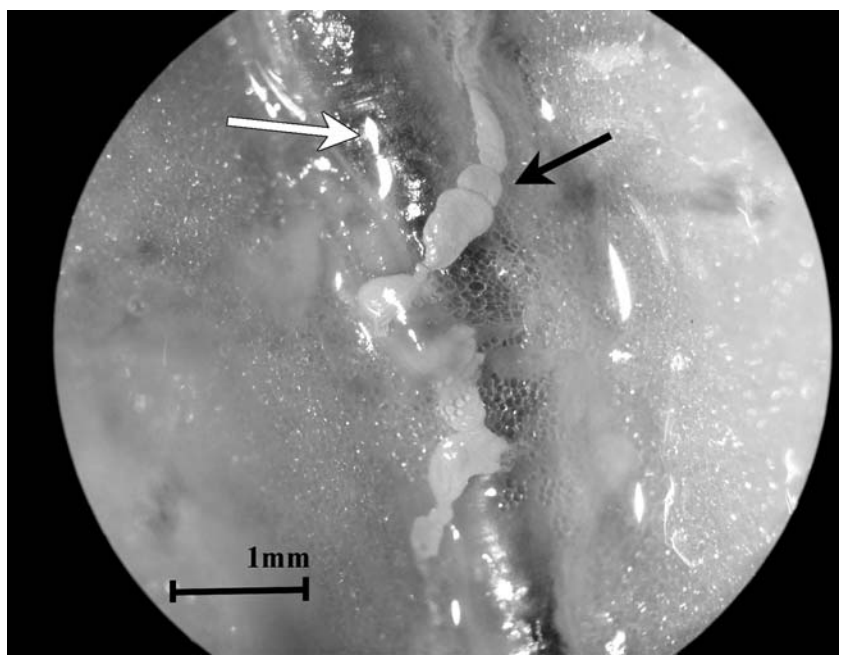

Fig. 10. A photograph of the perforating lymphatic vessel (*black arrow*) along the branch of the internal mammary artery (*white arrow*). (From Suami H, et al. The lymphatic anatomy of the breast and its implications for sentinel lymph node biopsy: a human cadaver study. Ann Surg Oncol 2008; 15:883–871. Reprinted with permission).

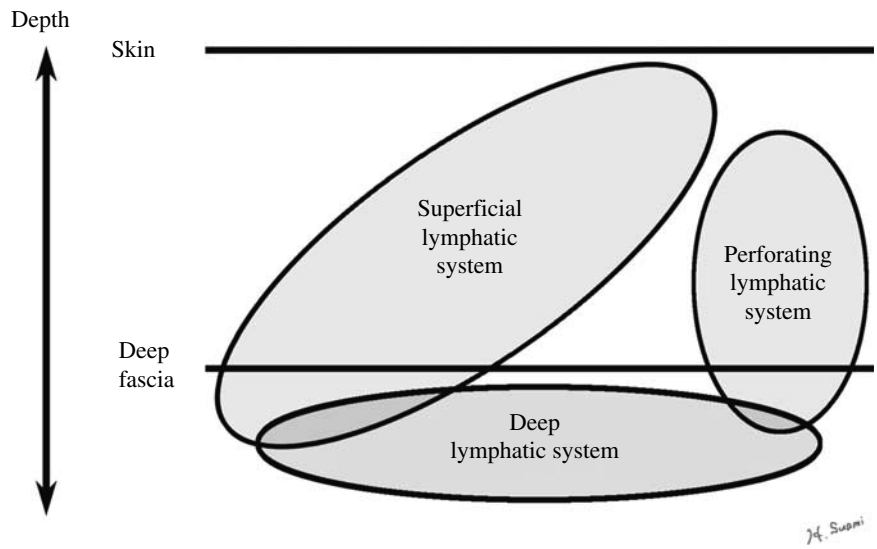

Fig. 11. The authors' concept of the breast lymph drainage, drained by both the perforating lymphatic system and the conventional horizontal superficial lymphatic system with their relationship to the deep lymphatic system beneath the deep fascia. (From Suami H, et al. The lymphatic anatomy of the breast and its implications for sentinel lymph node biopsy: a human cadaver study. Ann Surg Oncol 2008; 15:883–871. Reprinted with permission).

6. CLINICAL IMPLICATIONS FOR SENTINEL NODE BIOPSY

Lymphatic mapping with lymphoscintigraphy and the sentinel node biopsy have become a standard procedure for assessing the advance of breast cancer. Controversy exists over injection sites of dye and/or radioactive tracer among peritumoral, subareolar, and intradermal injection for accurate lymphatic mapping. Our anatomical findings suggest that if there is only one sentinel node draining the entire breast, then there is no discrepancy between the injection sites (Fig. 8D). However, in the case of multiple sentinel nodes, it is more problematic. Colour-coded diagram Fig. 8B shows that, if a primary breast tumor is located in the vicinity of the purple lymph vessel (lower outer quadrant), there is the possibility of cancer cells reaching the dominant green sentinel lymph node as well as the orange node because there is a connection between these two nodes. If the tracer is injected only into the subareolar regions, it will reach the green sentinel node but may miss detection in the orange sentinel node because numerous valves regulate lymph flow and stop its backflow. In this study, the purple lymph vessel courses in the deep layer passing through the breast tissue so that a superficial intradermal injection will not be taken up by this vessel (Fig. 9, left). These anatomical analyses suggest that the peritumoral injection is recommended for accurate lymphatic mapping.

7. CONCLUSIONS

Historically, there are very few reports regarding the gross anatomy of the lymphatic system of the breast. Current anatomical knowledge is based on the results of anatomists, Sappey, Cuneo, and Poirier obtained more than 100 years ago. These findings provide much needed important information of the lymphatic drainage of the breast.

It is well known that the lymph-collecting vessels are the main route for carrying cancer cells from primary tumor sites to regional lymph nodes. Accurate knowledge of lymphatic anatomy

for predicting cancer metastases is essential for clinicians. This refined protocol has enabled us to demonstrate the breast and anterior upper torso lymphatic drainage in adult male and female cadavers. This anatomical analysis suggests that a peritumoral injection with a radioactive tracer is recommended for tracking down lymphogenous tumor spread to the sentinel node biopsy.

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6 Should Axillary Lymph Node Dissection be Done for Breast Cancer?

Douglas Reintgen, MD, Blake Cady, MD, and Stanley P.L. Leong, MD, FACS

CONTENTS

ABSTRACT

Selective sentinel lymphadectomy or sentinel lymph node (SLN) biopsy has been shown to be an excellent and minimally invasive staging procedure for early breast cancer with no clinical adenopathy. When the SLN is negative, no axillary lymph node dissection (ALND) is needed. The debate becomes active as to what should be done when the SLN is positive. Dr. Reintgen presented the pro view that complete ALND should be done whereas Dr. Cady presented the con view.

Key Words: breast cancer; SLN biopsy; ALND

1. INTRODUCTION

The potential role of axillary lymph node dissection (ALND) in early breast cancer consists of staging the patient, regional control, and perhaps survival. It has been well established that lymph node status is the most important predictor of patients' clinical outcome. Therefore, ALND has been considered to be an excellent staging procedure *(1)*. However, the therapeutic role of the ALND has not been well established. In the sentinel lymph node (SLN) era, selective sentinel node procedure has become standard for staging the axillary *(2–4)*. The critical question is whether patients require complete ALND if the SLN(s) are positive. For that reason, the American College of Surgeons Oncology Group (ACOSOG) launched a protocol in 1999, Z0011, a randomized trial

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of ALND in women with clinical T1-2NOMO breast cancer who have a positive SLN to have complete ALND or not. Unfortunately, the study has prematurely been terminated because of poor accrual. Therefore, without the data from this important study, the debate goes on whether a patient should have a complete ALND upon the diagnosis of a positive SLN.

2. AXILLARY NODE DISSECTION IN PATIENTS WITH INVASIVE BREAST CANCER: PRO BY DOUGLAS REINTGEN

Of course, if the SLN is negative, there is no need for ALND. However, there are really two questions to address the issue of whether to perform an axillary nodal dissection in women with invasive breast cancer. The first is whether there is a population of women with invasive breast cancer who do not need any nodal staging surgery and can be best treated with either simple mastectomy or lumpectomy and radiation therapy alone. Certainly patients with a small low-grade breast cancer, like a tubular cancer, probably do not need their regional basin addressed. However, this may cover only about 1% of the invasive breast cancers and a strategy needs to be developed for the remainder. The second question that needs to be addressed is if the SLN procedure is performed and the SLN is positive for metastases, does a completion or therapeutic lymph node dissection (CLND or TLND) need to be performed? Is there a survival benefit associated with this surgical strategy?

Lymphatic mapping and SLN biopsy have revolutionized the surgical treatment of melanoma and breast cancer. Very quickly this procedure became the standard of care for the treatment of both cancers due to the win/win situation available with the technique. SLN biopsy allows for more accurate staging with serial sectioning, immunohistochemical staging, and even molecular techniques to identify the low-volume metastatic disease that can be present in the SLN. In addition, SLN biopsy has proven to be a less morbid procedure when compared with the past method of nodal staging, the level I and II axillary node dissection. Since the field of lymphatic mapping for melanoma is 5 years ahead of the field of lymphatic mapping for breast cancer, and since the tumors are comparable in regard to surgical treatment if not biology, some hints of outcome can be gleamed from the more mature melanoma data.

The Intergroup Melanoma Trial of Balch *(5)* randomly assigned patients with no clinical evidence of nodal disease to an elective lymph node dissection (ELND) or observation and a TLND if clinically evident disease occurred. This trial was unique in that it used preoperative lymphoscintigraphy to define all nodal basins at risk for metastases and required the surgeons on the trial to dissect all basins identified to be at risk by lymphscintigraphy. In addition, the design of the trial was different in that it provided for prospective stratified groups that could later be analyzed separately. A survival advantage was seen for patients with nonulcerated melanomas, patients with melanomas between 1 and 2 mm and extremity melanomas. This trial strengthens the argument that removing the disease when it is microscopic has a survival advantage in select patients. The controversy of whether ELND contributed to a survival benefit in patients with melanoma is an academic question at this time due to the rapid incorporation of the SLN procedure. Lymphatic mapping and SLN biopsy have been shown to be the most accurate methods of obtaining nodal staging information. Approximately 10–15% of patients with either melanoma or breast cancer are upstaged with the more detailed examination of the SLN.

The SLN procedure is the most accurate staging method in both the node-negative and node-positive directions. Dessureault and colleagues *(6)* review a multi-institutional database of 14,914 node-negative melanoma patients. Retrospective analysis found a statistically significant survival advantage in patients with melanoma greater than 1 mm who were staged to be node negative with the SLN biopsy. Five-year survival rates were 90.5, 77.7, and 69.8%, respectively,

for patients with node-negative disease staged by SLN biopsy, ELND, or clinical examination alone. This supports the ability of the SLN biopsy to upstage patients traditionally deemed node negative and identify a select group of patients with a high likelihood of cure by surgery alone, no matter what their tumor thickness or ulceration status is. The malignant phenotype of a cell that is able to leave the primary site, survive in the lymphatic circulation, invade a foreign area like the parenchyma of a regional lymph node, and survive and grow is certainly ominous. If one is able to analyze the SLN, the most common site for metastases, in a detailed fashion and there are no signs of metastases, then that patient is closed to being declared cured of their cancer. The Dessureault study also supports the general feeling that even with an ELND, pathologic evaluation of the specimen often fails to identify and underestimates the number of patients with micrometastatic disease. The challenge then becomes to determine whether the missed micrometastatic disease, missed by routine histology, is clinically relevant disease. Mocellin and colleagues recently reported a meta-analysis of 22 studies enrolling 4,019 patients who underwent SLN biopsy for clinical Stage I and II melanomas *(7)*. These authors report that the PCR status of the SLN (assay with sensitivity of being able to identify 1 melanoma cell in a background of 1 million lymphocytes compared with routine histology's ability to identify 1 melanoma cell in a background of 10,000 normal cells) correlated with TNM stage, disease recurrence ($p < 0.0001$), and survival ($p = 0.002$) to suggest that the upstaging that occurs with a more sensitive assay for metastatic disease is clinically relevant disease.

The Multicenter Selective Lymphadenectomy Trial (MSLT) *(8)*, headed by Morton and colleagues, addressed the survival benefit associated with this surgical strategy. This 2,000-patient randomized, multi-institutional trial identified a 19% incidence of positive SLN biopsy results. This is essentially equal to the 18% incidence of clinically evident regional basin disease in the control group whose nodal basin was observed. The findings also showed a disease-free survival advantage for patients with positive SLNs who had an immediate completion lymph node dissection compared with those observed and later developed clinically evident regional disease despite undergoing a TLND. There is also evidence that progressive disease occurs if microscopic nodal disease is left in place. In the MSLT study, if the TLND was performed immediately after a positive SLN biopsy, then the mean number of involved nodes was 1.4. However, if the nodes were initially observed and then became palpable and the patient underwent a TLND, then the mean number of nodes with metastatic disease was 3.3. In a European study *(9)*, those patients who had a false-negative SLN biopsy and later recurred in their regional basin were characterized by a higher ratio of two or more metastatic nodes and extracapsular involvement of lymph nodes after a LND compared with those patients who had an initial positive SLN followed by an immediate CLND. For these two studies, it is clear that micrometastatic disease in the regional basin, if left in place, will progress and cause a deterioration of survival.

The SLN procedure for melanoma proves to be the least morbid procedure for obtaining the nodal staging information in melanoma. In the SunBelt Melanoma Trial (SBMT) *(10)*, a total of 2,120 patients were evaluated with a median follow-up of 16 months. Overall 96 (4.6%) of the 2,120 patients developed major or minor complications associated with SLN biopsy, whereas 103 (23.2%) of the 44 patients experienced complications associated with SLN biopsy plus CLND. The most common complications associated with SLN biopsy were hematoma/seroma formation (2.3%) and wound infection (1.1%) The most common complications seen with CLND were lymphedema (11.7%), wound infection (7.0%), hematoma/seroma (5.9%), and sensory nerve injury (1.8%).

What about breast cancer? The SLN procedure proves to be the least morbid and the most accurate method to determine the status of the regional basin. Wilke and colleagues *(11)* reported on the complications of performing the SLN procedure in women with breast cancer. Over 5,500 subjects were entered from 1999 to 2003 into a prospective multicenter trial performed by the ACOSOG designed to evaluate the prognostic significance of micrometastases in

the SLNs and bone marrow in women with early-stage breast cancer. The sister trial to this Z-10 study then randomized patients with a positive SLN to either CLND and adjuvant therapy or just adjuvant therapy to define whether all women with a positive SLN need to undergo a CLND. Although this part of the trial was stopped prematurely before accrual goals were reached, it is hoped that the patients randomized will be sufficient to eventually answer the question of efficacy. In the patients who received blue dye alone or in combination with radiocolloid, a mild to moderate allergic reaction to the blue dye was reported in 0.6% of the patients, and anaphylaxis was reported in 0.1% of the subjects. Increasing age and increasing number of SLNs removed were associated with an increasing incidence of axillary seroma. At 6 months, 8.6% of the patients reported axillary paresthesias (mild in >90%), 3.8% had decreased upper extremity range of motion as compared with baseline, and 6.9% demonstrated proximal upper extremity lymphedema. Significant predictors of 6-month surgical complications included decreasing age for axillary paresthesias and increasing body mass index and increasing age for upper extremity lymphedema. Significantly less complications were seen in patients who had the SLN procedure compared with those that had the SLN procedure followed by a CLND.

The SLN biopsy is the most accurate method available to define both the node-negative and the node-positive population in breast cancer. The more detailed examination of the SLN with increased sectioning and immunohistochemical staining will upstage about 10–15% of the node-negative population. For node-negative disease, women staged to be node negative with SLN procedure have a much better survival than those women staged to be node negative in the past with an axillary node dissection and a superficial histologic examination of the regional basin. *(12)* Clinicians have to realize that the node-negative population today will have a much better survival than in the past due to more accurate staging.

There is also evidence that missed micrometastatic disease in breast cancer is clinically relevant disease in that eventually those patients upstaged with a more intense histologic exam will have a poorer outcome. In the International Ludwig International Breast Cancer Trial *(13)*, 1,000 women with invasive breast cancer and originally declared to be node negative with routine examination had their regional basin retrospectively analyzed with serial sectioning. Ten percent of this population was upstaged to node-positive disease with the more intensive examination and this upstaged population had a worse recurrence rate and overall survival. A later study by Cote and colleagues *(14)* showed that much of this survival difference was confined to post-menopausal patients. There has been 25 studies examining the clinical importance of micrometastatic disease in the SLN and six of the seven largest studies with the proper power showed survival differences for patients upstaged.

It is clear that performing a level I and II axillary node dissection after a positive SLN will remove more disease in neighboring non-SLNs. For H&E-discovered micrometastatic disease, approximately 40% of patient will have disease beyond the SLN. Even for breast cancer patients with less of a tumor burden in the SLN, such as those with N1mi (metastatic focus between 0.2 and 2 mm) or N0I+ (metastatic focus less than or equal to 0.2 mm) higher level disease in non-SLNs occurred 15 and 5% of the time, respectively *(15)*.

Is there a survival benefit associated with performing an axillary node dissection in breast cancer? Polednak *(16)* surveyed the SEER database of 69,543 patients with invasive breast cancer and node-negative disease. There was a significant higher risk of death in patients with 0, 1–3, and 4–10 nodes examined than in patients with 20 or more nodes examined, even in women with small tumors less than 2.0 cm. The study suggested that with an analysis of lesser number of nodes, patients are understaged and there may be a survival advantage associated with removal of occult node-positive disease. Further analyzing the SEER database shows that those women with invasive breast cancer and N0 disease have a 5-year survival of 90%, which was significantly better when compared with the 5-year survival of 86 and 82% in patients with N1mi and N1

disease, respectively. In another study, using Bayesian meta-analysis of six published randomized trials (ACOSOG, Copenhagen Trial, SE Scotland, GUY 1 and 2, NSABP B-04, and Institute Curie trial) *(17)* comparing standard treatment to standard treatment omitting ALND, Richard Orr showed that there was a subgroup of patients, perhaps 5%, who had an increased survival because of axillary node dissection, most pronounced for those with Stage I and II disease. This survival benefit was noted to be independent of any addition systemic or regional therapy received. The reduction in survival by omission of ALND was 7–46%.

The final word as to whether ALND contributes to a survival benefit in women with invasive breast cancer will be answered by two prospective randomized studies, the ACOSOG Z-11 study and the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-32 trial. Until these trials are mature, the more accurate staging, the less morbidity, the data to suggest missed micrometastatic disease are clinically relevant disease, and the suggestion that axillary dissection contributes to a survival benefit all point to the following conclusions. SLN biopsy should be performed in all women with an invasive breast cancer and if the patients have a positive SLN, then a CLND should be performed.

3. AXILLARY NODE DISSECTION IN PATIENTS WITH INVASIVE BREAST CANCER: BY DR. BLAKE CODY

Dr. Reintgen has given his approach regarding the necessity of ALND in invasive breast cancer. My role is to contest that approach with arguments and rationale to support my position.

Dr. Reintgen cites literature other than breast cancer, but I would point out that the breast cancer field is mature and voluminous enough to rely on its own literature, since articles regarding lymph node metastases, SLN biopsy, and the outcome of varieties of regional lymph node resections are more than ample to answer any questions in this particular cancer *(18,19)*. There is no necessity to bring in other malignancies and their lymph node metastases to support arguments regarding breast cancer, although I too will cite some in this chapter.

Adjuvant systemic therapeutic decisions in invasive breast cancer now relies more on primary tumor features rather than nodal pathology. In addition, the frequently repeated concept that "axillary lymph nodes are the principal prognostic feature in invasive breast cancer" is no longer tenable, because of the increasing use of genetic analyses of breast cancers, such as the Oncotype DX *(20,21)*. These sophisticated genetic analyses are more discriminatory and significant for judging outcome and systemic therapy selection than size, grade, or lymph node metastases in breast cancer. Because of the current presentation of breast cancer in heavily screened populations, less than 25% of patients now have any lymph node metastases in the axilla, even utilizing SLN biopsy technology and more careful pathology examination *(22)*. In roughly 50%, or even more, of patients with axillary lymph node metastases, all the metastases are contained within the few nodes removed by SLN biopsy *(23)*. In contrast to a decade or two ago, the number of lymph node metastases does not govern the selection, duration, style, or components of systemic chemotherapy regimens nor the particular hormonal regimens recommended for patients. Medical oncologists choose the apparently most effective adjuvant chemotherapy or hormonal therapy regimen regardless of the number of nodes, and even regardless of the prognosis determined by other features. The contemporary attitude is that if an adjuvant systemic therapy is needed, it should be the most effective available and should apply to patients with poor prognostic features but negative nodes, patients with one node metastases, or patients with multiple positive nodes. Another aspect of the current discussion about axillary lymph nodes is the fact that, with breast conservation and radiation therapy, the lower two-third of the axilla is treated with the breast tangent radiation fields and receives an adjuvant therapeutic dose of

radiation therapy resulting in very infrequent axillary nodal relapses *(24)*. This anatomic coverage by radiation has been documented in numerous studies. Thus, the concept that the axilla is not treated if lymph nodes, other than the sentinel node, are not removed is not true. This is demonstrated clearly by the fact that in undissected axillas from patients with a clinically negative axilla and breast conservation, the nodal recurrence rate is reported to be 2% or less *(25,26)*. As a matter of fact, even in patients who have a positive sentinel node, in the few studies that have addressed the issue, the failure to do a subsequent ALND again yields an axillary clinical nodal recurrence rate of less than 2% in short follow-up *(27)*.

Another issue of controversy in ALND is the biologically implausible concept reported in the literature and emphasized by Reintgen that indicates that if more negative nodes are removed, patients have better survival than if fewer nodes are resected. This concept flies in the face of the fact that in retrospective studies, randomized trials, and meta-analyses, increased survival cannot be documented regardless of how lymph nodes in the axilla are managed, whether by observation only, limited dissection, or more radical dissection *(18,19)*. These data apply both to patients who have a defined high rate of pathologically positive lymph nodes and to those who have negative axillary lymph nodes. Therefore, the concept that removing more negative nodes increases survival makes absolutely no sense whatsoever and is without any conceivable biological explanation except as a function of more accurate staging. The fact that greater survival is detected in the reported nonrandomized studies and retrospective studies that address this issue undoubtedly has to do with a "Will Rogers" phenomena of improved staging by more accurate or greater tissue analysis or stage shifting. There undoubtedly are other issues of patient selection that have led to these irrational conclusions, but it cannot be a biological explanation that this occurs when randomized trials comparing various surgical approaches to the axilla that does contain metastases do not demonstrate a survival advantage.

Orr, in a meta-analysis of five randomized trials published a few years ago, maintained that there is a 5% increase in survival in patients who had axillary dissection in contrast to no dissection *(28)*. This meta-analysis was flawed by the fact that one of the five trials was from the Institute Curie, which at the 5-year report noted that there was a statistically significant improvement in survival, but this advantage disappeared by the 8-, 10-, and 15-year analyses of the same Curie trial data *(29)*. The apparent initial survival advantage, as reported by the Curie authors, was due to the administration of adjuvant systemic therapy to those patients in the axillary dissection arm who had positive nodes, which was not administered to patients in the nondissected axilla arm since no positive node determination could be made. We have recently completed two extensive reviews of evidence-based literature including randomized trials, meta-analyses, and retrospective studies of good quality; no survival improvement in any organ with epithelial cancer that is subject to varieties of regional nodal dissection could be demonstrated *(18,19)*. Thus, cancers not only of the breast but also of the esophagus, lung, stomach, colon, rectum, and melanoma demonstrated no consistent overall survival advantage in any report. Our first study examined the literature up to the year 2000 *(18)*, and the more recent report summarized the literature between the years 2000 and 2006 *(19)*.

It may be that microscopic metastatic disease seen in SLNs, either in the peripheral sinus or in the substance of the lymph node, if larger than 0.2 mm may be prognostically relevant, as indicated by a number of studies; however, the real issue is whether treatment by ALND of such patients produces a survival benefit. For that analysis, there is not evidence that there is difference in overall survival, albeit a statistically significant difference in axillary recurrence does occur by the addition of ALND after the SLN biopsy. An aspect of this conclusion, of course, is that many patients with a negative SLN biopsy, or a negative ALND, still die of metastatic disease. The statement by Reintgen that if the sentinel node is negative, they are "declared cured of their cancer" is completely wrong. His conclusion is a misinterpretation of evidence and data

regarding curability of breast cancer. As in the remote past, some patients with negative nodes still die of disease, and the fact that the disease burden in the axillary nodes is less in recent years does not indicate that patients who are free of SLN metastases have no risk of metastatic disease in vital organs. One of the fascinating aspects of breast and other cancers is that even with extremely large cancers and other poor prognostic features, the rate of axillary nodal metastases never approaches 100%. In summary SEER data, even cancers greater than 8 cm in diameter never have an axillary lymph node metastases rate of greater than about two-third *(30)*. Exactly similar data were reported by us many years ago in colorectal cancer, where again it was noted that even with large and advanced colorectal cancers from earlier periods there were never more than about two-third of patients who had positive lymph nodes *(31)*. This substantiates the conclusion that there are "lymphotrophic" or "lymphatic seeking" organ-specific metastatic cancer cells, in contrast to a proportion of cancers that, even at their largest and with the worst prognosis, do not shed cells that are lymphotrophic; indeed such circulating metastatic cells may have a lymphatic avoidance behavior, yet still the patients have a poor prognosis and many die of disease. This again points out the organ site specificity of metastatic disease, which is displayed in animal model research reports as well as in clinical studies, demonstrating liver-only metastatic behavior as in colorectal cancer, carcinoid and pancreatic islet all tumors, and ocular melanoma and lung-only metastases in sarcoma and colorectal cancers *(23)*. The differentiated thyroid cancers display frequent cervical node metastases, yet have a 99% 20-year survival, so that these patients display a "lymph node only" metastatic pattern unrelated to outcome *(32)*.

The statement that the "most important prognostic factor" is axillary metastases in breast cancer certainly is no longer true. With genetic analyses of breast cancers displaying actual gene constellations, it is clear that genetic analysis now provides the most important prognostic features *(20,21)*. For instance, in the pioneer Vant Veer report, the 76 gene pattern described was more prognostically important in predicting survival than cancer size, lymph node status, or grade *(21)*. Thus, we are entering a new era where the lymph node metastases will not be an important prognostic feature, but will be just one of several clinical or morphologic features; eventually, the lymph node metastatic prognostic correlation will be subservient to the actual genetic analysis of individual patient's cancers.

A contemporary understanding of the lymphatic system must be based on the appreciation of its evolutionary development, physiology, and anatomy *(23,33)*. The lymphatic system developed and became more sophisticated through time as a result of evolutionary adaptation solely as a defense mechanism against foreign antigens such as bacteria, viruses, parasites, and toxins *(23,33)*. The collection of immune competent lymphocytes in lymph nodes interspersed in the lymphatic stream was the eventual method of analyzing antigens from the surface of the body to enable production of both humeral antibodies and cytokine-mediated cytotoxicity in the lymphatic-based sophisticated adaptive immune system *(34)*. The adaptive immune system and indeed the earlier innate immune system had nothing to do with the analysis of or protection from cancer, since cancers by and large are more "self" than "other" and generally an abnormal cellular development of the aging organism, but not a frequent phenomena in young organisms which need preservation, evolutionarily, through their reproductive life. Therefore, lymphatic involvement with cancer detected by removing a small regional portion of the lymphatic system and lymph nodes as an adjacent component of the primary organ removal has always been purely incidental to the basic immunologic function of the lymphatic system. This can be particularly emphasized by recent organ transplantation literature which notes that transplanted organs can convey cancers to immunosuppressed recipients when the donor has had invasive cancers of a variety of organ sites, including even glioblastoma *(23)*. These organs containing dormant metastatic cells can transplant cancer cells from the apparently disease-free donor to the immunosuppressed recipient. Such latent or dormant cancer cell clusters can be transported by

transplanted kidneys, hearts, lungs, and livers. This literature that demonstrates the transplantation of cancer cells along with the donor organs merely emphasizes that microscopic collections of cancer cells lie not just in the lymph nodes, which are found because of lymph node harvesting and analysis, but in the bone marrow which can be readily sampled and also in kidneys, livers, hearts, and lungs which are never sampled. These dormant cancer cells create lethal metastatic cancers in immunosuppressed recipients in as high as 45% of heart and lung donors who have previously had a wide variety of invasive cancers *(23)*. Undoubtedly this phenomenon of dormant cancer cells becoming active is a function of the paralysis of the immuno-surveillance system that contains and controls dormant cancers up to 2 mm in diameter before the angiogenic acquisition of blood supply. It is an accident of surgical resection of adjacent tissue that we react to cells in the regional axillary lymph nodes, while we cannot react to cells that are obscure and would have been found only if similar sophisticated tissue analysis was done of bone marrow, liver, heart, lung, and kidney. We react to the cells in the lymph nodes because we find them, whereas we do not react to cells that might have been found in these other organ sites merely because we do not analyze them with the similar sufficient rigor that we utilize to analyze lymph nodes.

Our meta-analysis of recent trials of axillary treatment in contemporary breast cancer has demonstrated no survival advantage whatsoever while also displaying statistically significant differences in axillary nodal clinical recurrence, again emphasizing the lack of control of survival by lymph node metastases or regional lymph node treatment, while acknowledging the presence of small collections of cells in lymph nodes *(35)*. The concept that we need to wait for the results of the aborted ACOSOG Z-11 trial or the NSABP B-32 trial to conclude whether there is a survival advantage from axillary dissection in breast cancer patients is completely unnecessary since there are ample numbers of historical reports, evidence-based randomized trials, and meta-analyses, to thoroughly document that there is no survival advantage in breast cancer *(18,19)*. There is no survival advantage produced by regional nodal resections in epithelial cancers of a variety of other organs when reviewed systematically *(18,19)*. Lack of survival advantage by nodal resection in breast cancer in particular, of course, has been demonstrated for decades by trials conducted many years ago but considered now to be of insufficient power and size to prove the point.

In summary, in response to Dr. Reintgen's argument suggesting the advantages of ALND in improving survival in breast cancer, I conclude that none of his arguments are supported by data, not only in breast cancer but also in all other cancers, and this lends support to my denial of his point. As noted, our two recent analyses of all the evidence-based trials addressing this issue in a variety of human epithelial cancers fails to consistently demonstrate any survival advantage. Thus the initial question of whether axillary dissection should be utilized in breast cancer is, we believe, truly moot. As proposed over 20 years ago, lymph node metastases are "indicators, not governors," of outcome in breast and other cancers *(36)*. This biological theory and subsequent research, both clinical and laboratory, reemphasizes this point, and literature reviews summarize the arguments presented.

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II Tumor Microenvironment and Proliferation

7 Overview of Tumor Cells and the Microenvironment

Richard E.B. Seftor, PhD, Elisabeth A. Seftor, BS, Lynne-Marie Postovit, PhD, and Mary J.C. Hendrix, PhD

CONTENTS

ABSTRACT

The bidirectional communication between cells and their microenvironment is integral to both cancer progression and embryological development. This chapter illuminates major breakthroughs in our understanding of tumor cells and their microenvironment, the development of innovative experimental models that allow the study of targeting molecular signals in the tumor cell microenvironment, and finally, exploiting the convergence of embryonic and tumorigenic signaling pathways as a new therapeutic strategy for inhibiting tumor progression.

KeyWords: tumor microenvironment; multipotent tumor cells; Nodal; Lefty

1. TARGETING MOLECULAR SIGNALS IN THE TUMOR CELL MICROENVIRONMENT

Cancer is a disease of the tumor–host microenvironment consisting of a complex dynamic relationship. Based on recent molecular evidence revealing the presence of plastic, multipotent stem cell subpopulations within melanoma tumors, our studies have focused on the role of the microenvironment in potentially reprogramming these tumor cells toward a less aggressive phenotype *(1)*. To initiate these studies, we developed a 3D model to study the epigenetic effects on multipotential metastatic melanoma cells induced by the microenvironment of human embryonic stem cells (hESCs) *(2)*.The data revealed that amelanotic melanoma tumor cells exposed to the

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embryonic stem cell microenvironment were induced to form melanoma spheroids and reexpress the melanocyte-specific marker Melan-A. In addition, the embryonic stem cell microenvironment resulted in tumor cells that were less invasive in vitro and less tumorigenic in vivo.

In determining what factors might be responsible for the reversion of the tumor cells' metastatic phenotype, we focused our attention on Nodal, a potent embryonic morphogen and stem cell marker belonging to the transforming growth factor (TGF)-β superfamily which maintains hESC pluripotency and is overexpressed 20-fold in aggressive compared with poorly aggressive melanoma cells *(1)*. This is a unique observation since Nodal expression in humans is largely restricted to embryonic tissues (including trophoblasts), hESCs, and the developing mammary gland, but is generally lost in normal adult tissues. Furthermore, the extracellular Nodal inhibitors Lefty A (2) and Lefty B (1), also members of the TGF-β superfamily and critical in cell fate differentiation events, are expressed by hESCs, but not aggressive melanoma cells (Fig. 1). Using a multiplex polymerase chain reaction (PCR) analysis, we found Nodal expression knocked down by 87% in metastatic melanoma cells cultured on a matrix conditioned by hESCs (after removal of the hESCs), and metastatic melanoma cells exposed to hESC-derived Lefty (recovered from a matrix conditioned by hESCs) showed a decrease in Nodal protein expression concomitant with a decrease in their ability to form colonies in a clonogenic assay. We also found that addition of recombinant Nodal (rNodal) to the clonogenic assay could reverse the effect of the hESC-derived Lefty.

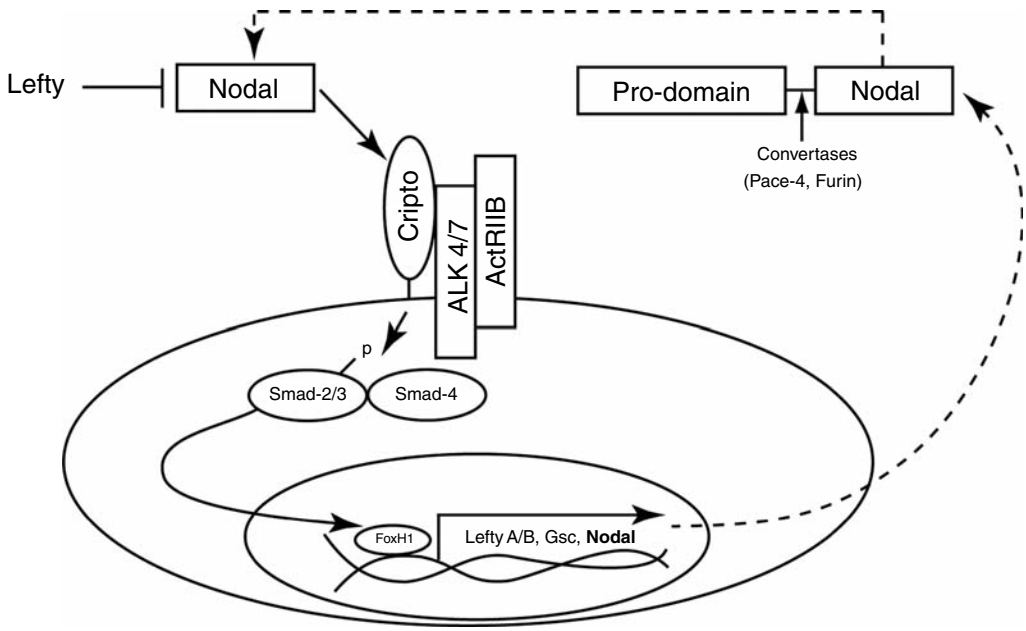

Fig. 1. A schematic overview of the Nodal signaling pathway. Nodal propagates its signal by binding to Cripto-1 and heterodimer complexes between type I (ALK 4/7) and type II (ActRIIB) activin-like kinase receptors. Assembly of this complex results in the phosphorylation and activation of ALK 4/7 by ActRIIB, ALK 4/7-mediated phoshorylation of Smad-2/3, association of Smad-2/3 with Smad-4, and then translocation of the Smad complex to the nucleus where it regulates gene expression through an association with transcription factors such as FoxH1, and Mixer—stimulating the transcription of Nodal and Lefty A/B. Cripto-1 directly associates with ALK 4 (with its CFC domain) and Nodal (with its EGF domain) for Nodal signaling. Nodal can bind to and activate ALK 7 in the absence of Cripto-1 and in its precursor form, can bind to ALK 4 in a Cripto-1-independent manner. Lefty A/B control Nodal signaling by spatially and temporally restricting the Nodal-mediated activation of ALK 4/7.

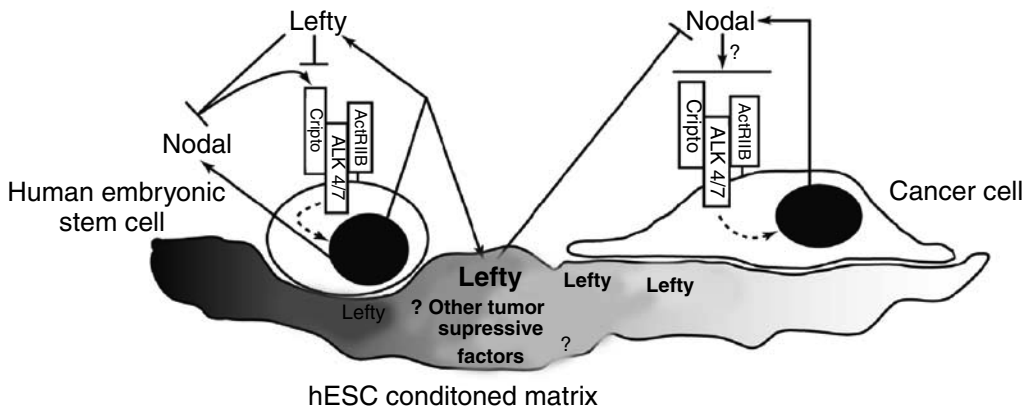

Fig. 2. Hypothetical model demonstrating how Lefty (and possibly other tumor suppressive factors), secreted by hESCs into their environment may reprogram aggressive tumor cells by inhibiting their Nodal signaling.

Subsequent sequence-based methylation analyses have provided some insight into possible mechanisms underlying the epigenetic reprogramming of the plastic, metastatic melanoma cells *(3)*. Although metastatic melanoma cells in the presence of the hESC microenvironment demonstrate only a 6.8% increase in the global methylation of CpG dinucleotides in CpG islands, there is a 32% increase in the methylation on the CpG dinucleotides in the CpG island associated with Nodal, possibly suggesting a silencing of this gene in tumor cells directly exposed to hESC-secreted Lefty (Fig. 2). By aligning such regions with the DNA sequence, we hope to elucidate whether these differentially methylated cytosines are associated with elements, such as transcription factor-binding sites.

Our studies have demonstrated that aggressive tumor cells manifest a functional plasticity and express multiple molecular phenotypes similar to pluripotent embryonic-like stem cells. We have shown that the microenvironment of hESCs, specifically via the secretion of Lefty, can induce a phenotypic change in aggressive melanoma cells, which may provide new therapeutic strategies for clinical intervention(s).

2. CONVERGENCE OF EMBRYONIC AND TUMORIGENIC PATHWAYS: ROLE IN TUMOR PROGRESSION

Bidirectional communication between cells and their microenvironment is integral to both cancer progression and embryological development. In addition, phenotypically plastic tumor cells, such as melanoma, share many characteristics in common with embryonic stem cells. Illustrative of this feature is the recent discovery that aggressive melanoma cells and hESCs both express Nodal, an embryonic morphogen belonging to the TGF-β superfamily *(1,4)*. As mentioned in the previous section, Nodal is responsible for maintaining hESC pluripotency, and also for initiating mesoderm formation, and orchestrating L-R patterning *(5)*. Also highlighted in the previous section, Lefty A (2) and Lefty B (1) are extracellular Nodal inhibitors, members of the TGF-β superfamily—critical in cell fate determination. Cripto (Cripto-1) is a coreceptor for Nodal and is an epidermal growth factor-Cripto-1/FRL1/Criptic (EGF-CFC) family member

(6). Nodal propagates its signal (Fig. 1) by binding to Cripto-1 and heterodimer complexes between type 1 (ALK 4/7) and type II (ActRIIB) activin-like kinase receptors. Assembly of this complex results in the phosphorylation and activation of ALK 4/7 by ActRIIB, followed by the ALK 4/7-mediated phosphorylation of Smad-2 and possibly Smad-3, association of Smad-2/3 with Smad-4 and then translocation to the nucleus where the Smad complex regulates gene expression through an association with transcription factors such as FoxH1 and Mixer—stimulating the transcription of Nodal and Lefty A/B.

Based on the multipotent potential of aggressive melanoma cells and their ability to express the embryonic morphogen Nodal, we asked whether these tumor cells could communicate with an embryonic microenvironment (in vivo), such as the zebrafish—and affect the development and fate of progenitor cells. Using this approach, our data showed that transplanted aggressive human melanoma cells—via the secretion of Nodal—can consequently induce ectopic cranial outgrowths and secondary body axes in zebrafish embryos *(4)*. Downregulation of Nodal expression in the melanoma tumor cells or overexpression of Lefty in the embryonic zebrafish abrogated these Nodal-induced effects on development. Furthermore, using antisense morpholino knockdown of Nodal expression or the SB431542 small-molecule inhibitor of the ALK 4/7 portion of the Nodal signaling pathway (Fig. 3) revealed that Nodal expression is regulated by a Smad-2-dependent positive feedback loop. Moreover, Nodal inhibition promotes the reversion of aggressive melanoma cells toward a less-aggressive melanocyte-like phenotype (Fig. 4), specifically downregulating the interconverted phenotype, abrogating vasculogenic mimicry potential, inhibiting clonogenic potential and tumorigenesis, concomitant with the emergence of melanocyte pathway-associated markers, such as Tyrosinase. These data illuminate a new pathway involving Nodal signaling, which plays a key role in melanoma plasticity and tumorigenicity, thereby providing a previously unknown molecular target for regulating tumor progression.

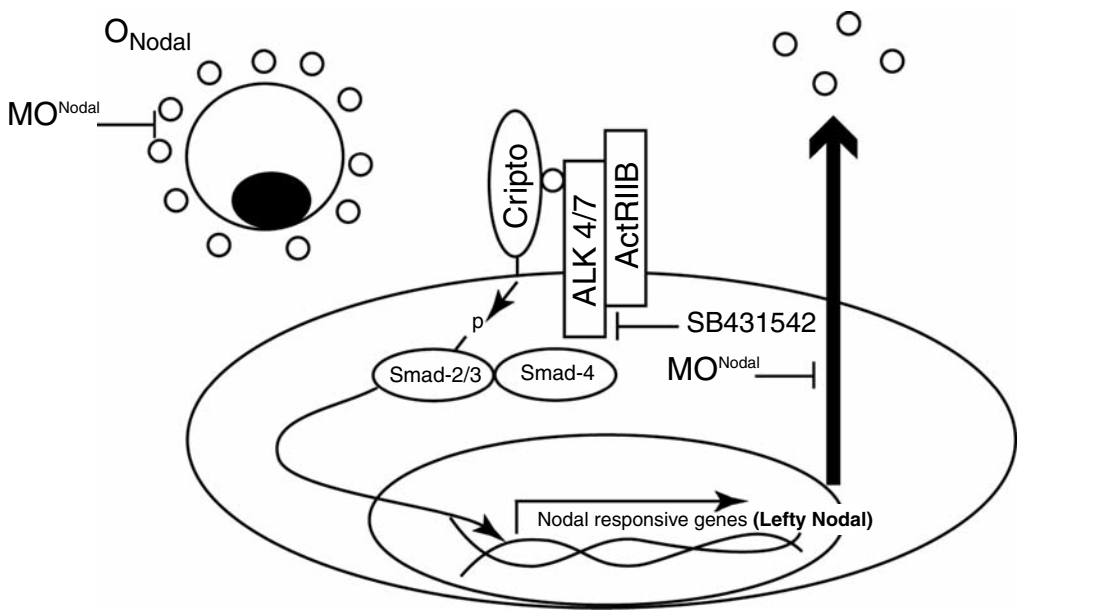

Fig. 3. Nodal expression is regulated by a Smad-2-dependent positive feedback loop. Nodal signaling may also be inhibited at the translational stage using morpholinos against Nodal, or by blocking the type I (ALK 4/7) activin-like kinase receptor with the SB431542 small molecular inhibitor.

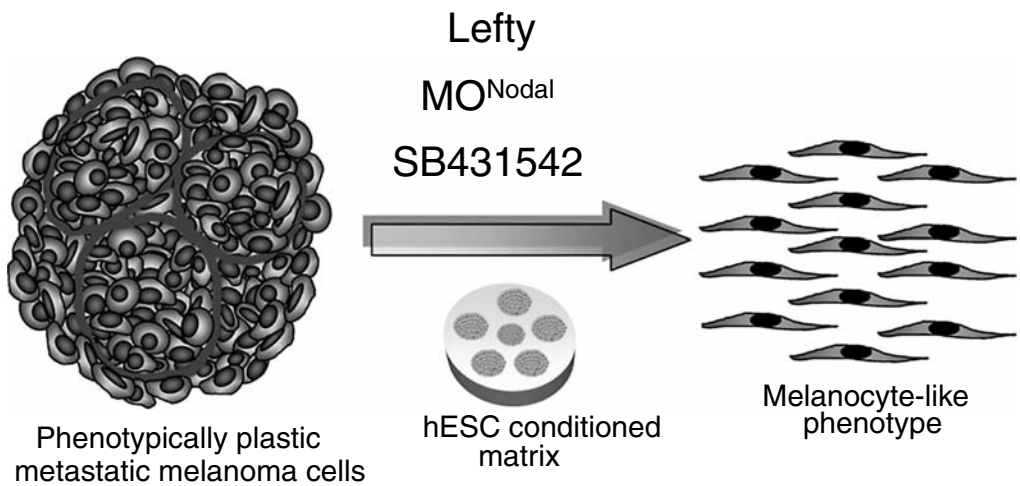

Fig. 4. Model for the exploitation of the convergence of embryonic and tumorigenic Nodal signaling pathways to reprogram multipotent metastatic melanoma cells to a melanocyte-like phenotype. Lefty is an inhibitor of Nodal; MO^{Nodal} is an antisense morpholino to Nodal; and SB431542 is a small-molecule inhibitor to ALK 4/7.

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III Lymphangiogenesis and Angiogenesis

8 Heme/Lymphvasculogenesis, Hem/Lymphangiogenesis, Hem/Lymphangiotumorigenesis, and Tumor Hem/Lymphangiogenesis: Need for a Terminology Adjustment

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Contents

ABSTRACT

The lymphatic and blood vasculatures develop and function as parallel yet interacting systems. In part because of the prominence of the latter and the dire sequelae of some blood circulatory disturbances, the lymphatic vasculature has been appropriately recognized only in lymphogenous spread of cancer and in congenital and acquired peripheral lymphedema. From the perspective of the last century's landmark contributions in basic and clinical lymphology leading to

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current understanding of structure–function relationships in the lymphatic system, coupled with recent advances in molecular lymphology, a terminology adjustment in the angiogenesis field is needed and indeed overdue. The prefix terms hem/heme- or lymph- should be used routinely to designate which of the two distinct vasculatures are "arising de novo" (vasculogenesis), "sprouting from preexistent vessels" (angiogenesis), "forming vascular tumors" (angiotumorigenesis), or "newly supplying or draining tumors" (tumor angiogenesis). Nondesignation of the prefix heme- or lymph- should be reserved for general reference to vasculatures or to an unknown or undetermined vasculature. In this presentation, the molecular and developmental pathways thought to be involved in these lymphatic and blood vascular processes will be summarized and specific clinical entities and examples along the spectrum of angiogenic disorders from benign to malignant highlighted to illustrate the importance of distinguishing the physiologic and pathophysiologic including neoplastic processes involving each of the two vasculatures and where they may interact, merge, or become indistinct and indistinguishable. Indeed, the success of targeted therapy directed at either or both depends on such understanding, clear terminology, and precisely addressing and articulating unanswered questions and unquestioned answers.

Key Words: lymphangiogenesis; hemangiogenesis; angiogenesis; vasculogenesis; angiodysplasia; angiomatosis; tumor angiogenesis; lymphedema; lymphatic development

1. LYMPHOLOGIC PERSPECTIVES

From the lymphologic perspective, (1) the lymphatic system is a vasculature similar to but distinct from the blood vasculature, a circulation (blood-lymph loop), absorptive apparatus, and the immune network ("lymphology" is the study of this integrated system of lymphatics, lymph, lymph nodes, and lymphocytes in health and disease); (2) interference (congenital or acquired) with the lymphatic arc of the blood–lymph loop circulation produces not just lymphedema (the specific study of which is "lymphedematology") but scarring, fat deposits, immunodysregulation, malnutrition, and angiogenic/dysplastic/tumorigenic disorders; (3) these pathophysiologic processes—linked by disturbed lymphatic vessel growth, that is, lymphangiogenesis (the study of which is encompassed in "lymphangiology")—intertwine with pathways and events in neoplasia and cancer growth and spread; and (4) lymphatic growth can be altered (lymphangiomodulation)—stimulated or inhibited—by a variety of external (e.g., pharmacologic agents) and internal factors and physiologic/disease processes—as well as prevented or stabilized by others (lymphangioprotection).

2. PREMOLECULAR ERA OF (HEM)ANGIOGENESIS AND (BLOOD) VASCULAR CELL BIOLOGY

In 1971, Judah Folkman's landmark observations and concepts on the relationship between blood vessel growth/supply and tumor growth opened up the new field of angiogenesis *(1)*. In the decades that followed, there was a surge of interest in blood vascular (particularly endothelial) cell biology in health and disease with potential applications to therapy of cancer and vascular, inflammatory, and other diseases.

Our own work led us quite by chance into this new field of (hem)angiogenesis and subsequently lymphangiogenesis (vide infra). We had been interested for several years in what we termed "ischemic therapy," specifically, reduction in organ function and hyperfunction by controlling organ blood supply *(2,3)*. Aware of sporadic efforts many

decades earlier to control endocrine (e.g., thyroid) hyperfunction by reducing the blood supply to the gland, we tested splenic artery ligation and embolization to control splenic hyperfunction yet preserve splenic immunoprotective function in patients with symptomatic hypersplenism. This approach was at least transiently successful and served as a forerunner to other more effective approaches, including our own, to splenic preservation as an alternative to splenectomy *(4)*. Interventional embolic therapy is now widely used in other conditions including cancer but has not to date been fully applied to reduce endocrine or splenic hyperfunction or to modulate and preserve organ function through circulatory control rather than ablation.

More than a century earlier, there was intense interest in the field of embryology concerning the origin and development of the blood vasculature. Meyer (1853) reported that blood vessels in tadpole tails could grow by sprouting from preexisting vessels, thereby providing one of the earliest descriptions of hemangiogenesis *(5)*. Later, Clark *(6)* demonstrated the importance of fluid flow in the patterning and remodeling of the blood vasculature *(6)*. However, it was not until 1920 that the existence of the hemangioblast, a precursor cell that gives rise to blood endothelial cells and hematopoietic cells of blood islands, was proposed *(7)*. The model of the development of the blood vasculature has grown considerably since these important discoveries [reviewed by Coultas *(8)*]. Hemevasculogenesis gives rise to blood vessels in embryonic and extraembryonic tissues from precursor cells called angioblasts and hemangioblasts, respectively. The primitive blood vessel network expands by hemangiogenesis and is later remodeled into a hierarchal vasculature. Thus, the coordinated control of hemevasculogenesis, hemangiogenesis, and remodeling is required for the proper formation of the blood vasculature. This control is mediated by specific growth factors and their cognate receptors [reviewed by Yancopoulos *(9)*].

3. PREMOLECULAR ERA OF LYMPHANGIOGENESIS AND LYMPHATIC CELL BIOLOGY

We were introduced abruptly to the "lymphangiogenesis" (we coined the word) side of angiogenesis in 1982 by the immediate challenge of a desperate young woman (Patient 1) *(10)*. She presented with a massive, rapidly expanding cervico-mediastinal lymphangioma extending from the neck to the diaphragm and associated with widespread bony lymphangiomatosis (Fig. 1)—an entity sometimes called "benign metastasizing" lymphangiomatosis or alternatively "disappearing bone disease" (Gorham syndrome). The first successful culture of human lymphatic endothelium was derived from this lymphangioma at virtually the same time that Johnston and Gnepp reported their own efforts in *(10–12)*. Subsequent patients with angiodysplasias (angiomatosis–angiotumorigenesis) involving blood vessels, lymphatics, or mixed of both offered further insight and impetus for us to examine the similarities and differences and interactions/overlap of blood vessels and lymphatics, normally distinct, particularly when developmental and proliferative processes go awry *(13,14)*. We postulated a central role for lymphangiogenesis in this constellation of overlapping lymphologic syndromes (Fig. 2) and proposed a terminology adjustment (vide infra).

More than a century earlier and neglected for many decades since, the phenomenon of lymphangiogenesis (but not the word) had been described in detail and studied meticulously with tools available at the time *(15,16)*. The subject aroused great interest and intense controversy, a portion of which we chronicled in the very first chapter/review on

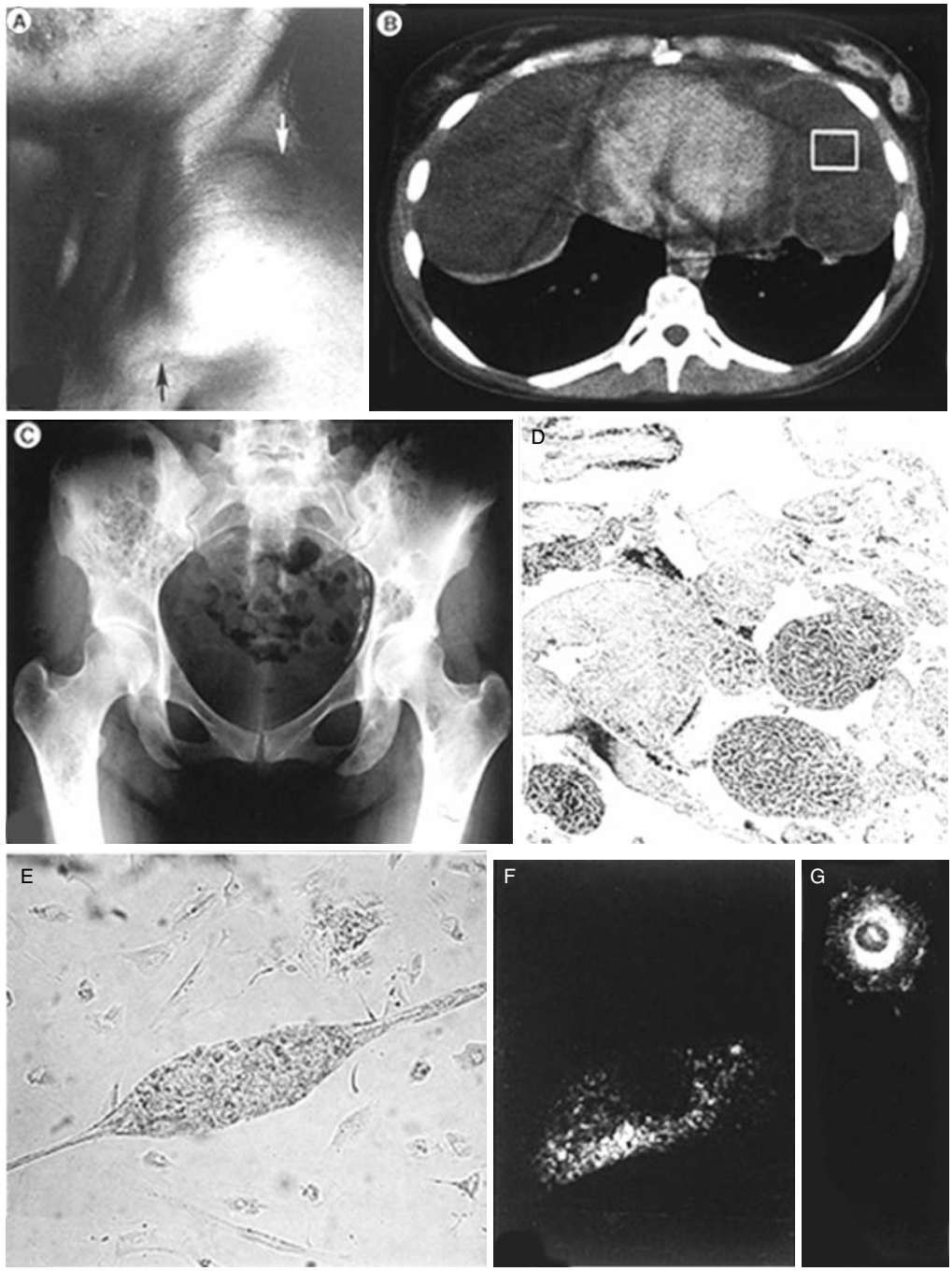

Fig. 1. Patient 1: 29 year-old woman with a rapidly enlarging giant cervicomediastinal lymphangioma (A, B) and diffuse osseous lymphangiomatosis (C). These lesions are rich in lymphatic tissue including lymphoid aggregates (D), surround and insinuate into adjacent tissues and critical organs (here, great vessels, lung and heart), and exhibit autonomous endothelial growth with tubule formation (lymphangiotumorigenesis in vitro) (E) with Factor VIII-related antigen (vWF) (F) and Ulex europaeus lectin (G) positivity on immunofluorescence. [See text for further details. Reproduced/modified with permission from Bowman C, Witte MH and Witte, C, et al, 1984 (10)].

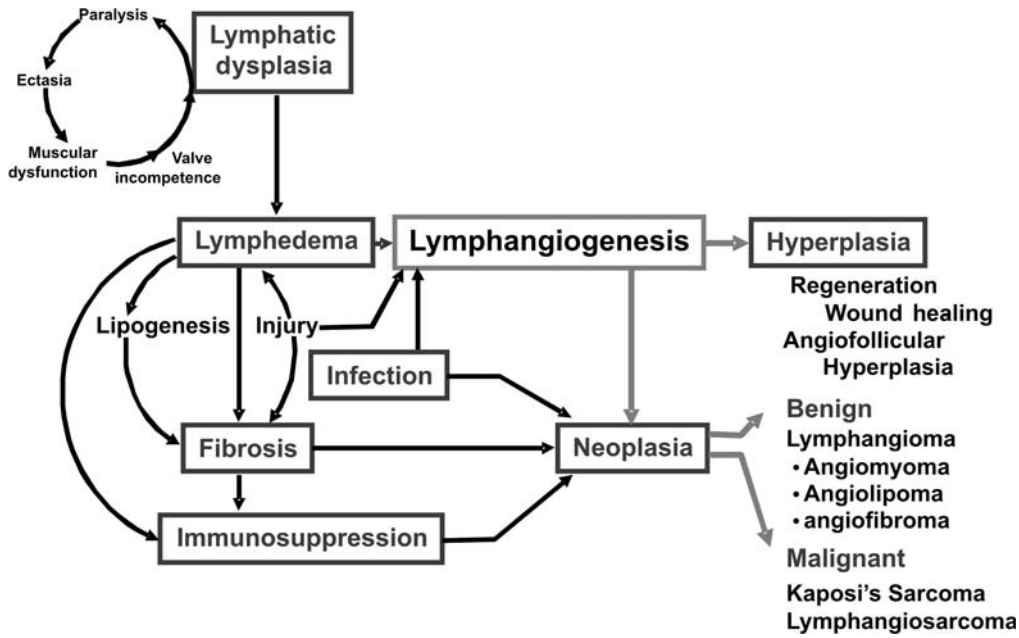

Fig. 2. Lymphangiogenesis and Lymphologic Syndromes. Flow chart illustrating the pathogenesis of peripheral lymphedema and some of its sequelae. According to this scheme, congenitally deficient or obstructed lymphatics promote lymph stasis, which is accompanied by deranged truncal contractility, progressive valve incompetence, destruction of contractile elements (lymphangioparalysis), and gradual ectasia of lymphatic collectors. After a variable period (occult lymphedema), sometimes aggravated by environmental trauma, a series of events is set into motion that culminates in chronic lymphedema. This clinical state is characterized not only by progressive swelling but also by fat and scar deposition, immunodysregulation, a propensity for infection, and microvascular proliferation; these processes, on the one hand, are essential for repair and regeneration but, on the other hand, may result in bizarre and poorly understood vascular new growths. (Reproduced with permission, Witte MH, Witte CL, 1986).

lymphangiogenesis which was published just before the opening of the current decade of molecular lymphology *(17)*. These historical landmarks in lymphangiogenesis in vivo (Fig. 3) and in vitro (Fig. 4) were documented in this publication, which appeared in 1997 and was widely cited for several years thereafter.

4. PROPOSAL FOR TERMINOLOGY ADJUSTMENT

In 1987, we proposed a terminology adjustment(Table 1 and Fig. 5) in the angiogenesis field to distinguish processes involving the blood vasculature from those involving the lymphatics *(18)*. To avoid anticipated confusion, clarify thinking, address research questions, and evaluate and treat patients, it was suggested that the heme/hem- or lymph- prefix be specified and the more general unprefixed term used for mixed conditions, unknown specificity, and general considerations. At that time, no specific vascular growth factors, receptors, and genes had yet been discovered for either vasculature; the focus was on human disorders of vascular growth (clinical phenotypes), animal models, and vascular cell biology, including the first successful efforts to culture and characterize isolated lymphatic endothelial cells *(10–13)*. This recommended terminology adjustment is still more relevant in the era of advancing molecular lymphology (vide infra).

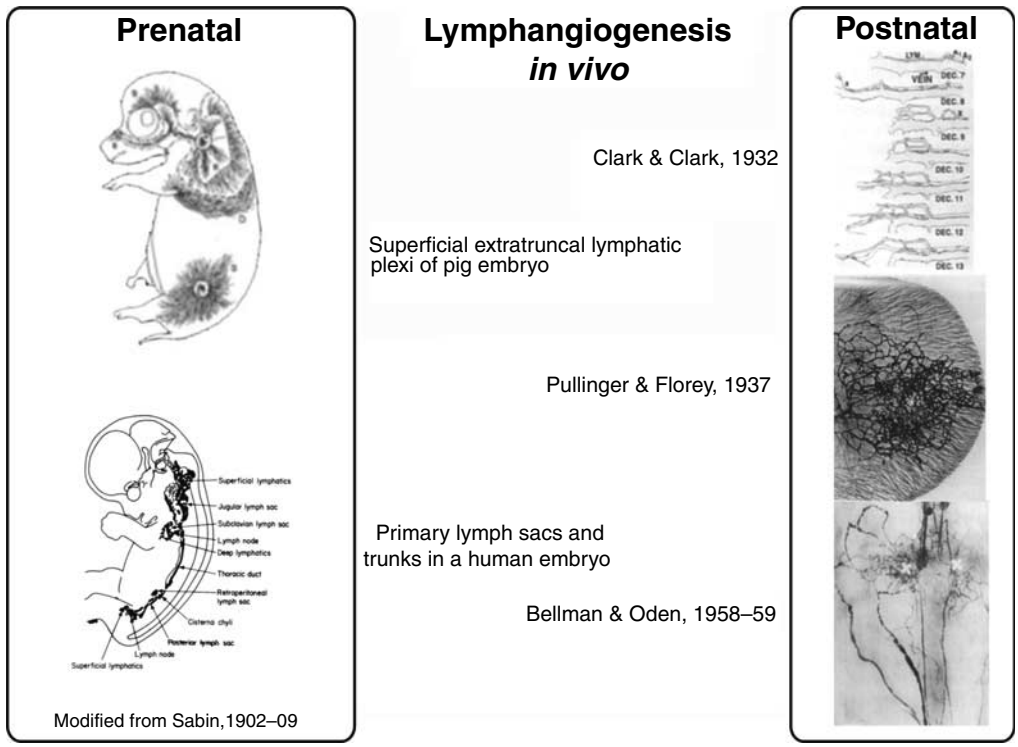

Fig. 3. Lymphangiogenesis In Vivo. *Left, top*: Superficial lymphatics in pig embryo. *Left, bottom*: Lymph sacs in human embryo (from Sabin 1902 to 1909). *Right, top*: Series of camera lucida, oil-immersion records, showing growth of an individual lymphatic capillary in the rabbit ear (LYM). Corresponding parts of a lymphatic and vein have been placed below one another in the drawings. *Right, middle*: Twenty-one days after making a turpentine abscess, which perforated a mouse ear, a dense new network of lymphatic capillaries surrounds the hole. *Right, bottom*: Micro lymphangiogram 24 days after a short transverse incision (*crosses*) in the skin of a rabbit ear. The distal part of the ear is uppermost. Several arcading vessels around the incision and numerous fine connections through the scar are seen (Reproduced/modified from Clark and Clark, 1932; Pullinger and Florey, 1937; and Bellman and Odén, 1958–59, respectively).

5. RESURGENCE OF INTEREST IN LYMPHANGIOGENESIS IN THE MOLECULAR ERA OF (HEM)ANGIOGENESIS

The past two decades have brought an explosion of interest and knowledge, at the molecular level, first about hemangiogenesis (and blood/hemevasculogenesis) then during the last decade also about lymphangiogenesis (and lymphvasculogenesis). First came the discovery of the chemical nature of (Hem)Vascular Permeability Factor by Dvorak *(19)* in 1983 and VEGFA by Ferrara in 1989 *(20)* (both presenters at this conference) along with VEGFA's cognate endothelial receptors, VEGFR1 and 2 (then known as FLK-1 and KDR). These events were followed by the discovery of the Angiopoietin family of vascular growth factors (beginning with Angiopoietin 1 and 2) by Yancopoulos *(21–23)* (also at this conference) and earlier, their TIE-1 and TIE-2 receptors, and the crucial role of this second pathway in blood vessel remodeling was established.

As mentioned earlier, in our first review of lymphangiogenesis in 1997 *(17)*, we had chronicled the century old history of the phenomenon of lymphvasculo/angiogenesis along with the prior decade's updates from the lymphatic cell biology era. The findings were linked to a conceptual

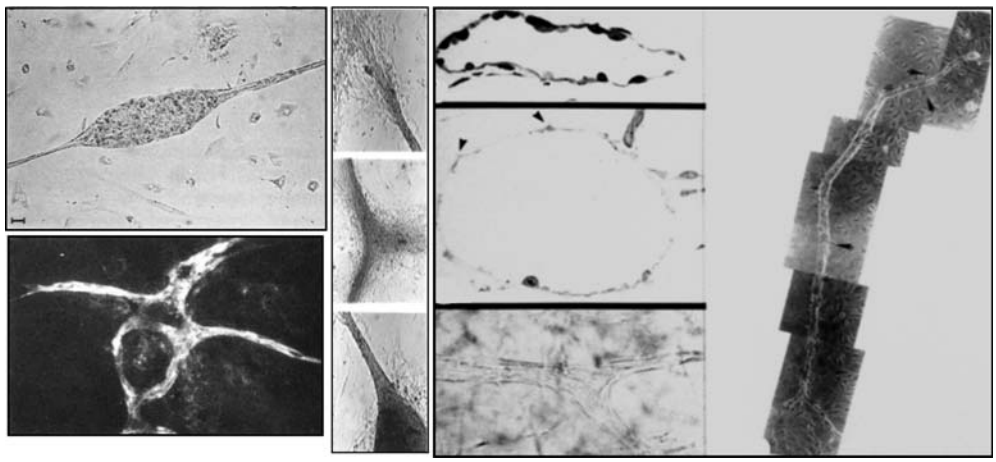

Fig. 4. Lymphangiogenesis In Vitro. *Left, top*: Primary culture of the cystic hygroma (lymphangioma) in Fig. 1 shows a mixture of cell types including bipolar spindle cells resembling fibroblasts and polygonal cells, which form bean-shaped nodules. *Left, bottom*: tubular structures observed throughout the medium brightly decorated with Ulex europaeus lectin (modified from 10). *Middle, left*: Spontaneous lymphangiogenesis in cell culture derived from a resected leg lymphangioma. Note loose clusters of lymphatic endothelial cells sprouting into branches (*top*) which are more prominent (*middle*) and evolve into a sheetlike aggregate with intense lymphatic-like sprouting branches (*bottom*). Reproduced/modified from Witte and Witte *(18)*. *Middle, right, top*: Hematic-like vascular channel lined by plump endothelial cells with *hump-shaped*, cross-sectional profiles in a 9-day-old plasma clot culture of a rat thoracic duct. Toludine blue stain (×1,000). *Middle, right, middle*: Newly formed, branching vascular channel in a 12-day-old living plasma clot culture of rat thoracic duct (×333). *Middle, right, bottom*: Light micrograph of lymphatic-like channel in a 25-day-old plasma clot culture of a rat thoracic duct. The highly attenuated endothelium is anchored to the surrounding fibrin by abluminal cytoplasmic filaments. Toludine blue stain (×928). Modified from Nicosia (1987). *Right*: One to three days after treatment with collagen type I, adjacent cells (*arrows*) continue to migrate and adhere to tubular structures with an increase in their dimension and length. Adhesion of cells (*asterisk*) at various points along the length of the tubular structures is accompanied by branching and the subsequent formation of elaborate capillary-like networks in the culture dishes. This lymphatic capillary tube that formed in vitro was derived from bovine lymphatic endothelial cells (×155) (Modified from Leak and Jones, 1994).

framework for lymphedema–angiodysplasia (LE–AD) disorders. Unbeknownst to us, this chapter was completed just prior to the discovery by Alitalo (also at this conference) of a specific member of the VEGF family with primarily lymphatic growth characteristics, namely VEGFC *(24)*, which was followed by VEGFD *(25)* and before both, their endothelial receptor VEGFR3 *(26–28)*. Since that time, other genes and proteins (Table 2 and Fig. 6) have been identified in the molecular cascade of lymphatic development based on both forward (mutant or transgenic mice) and backward (human family pedigree) genetic analyses. In addition to VEGFR3 mutations found in a subpopulation of Milroy familial lymphedema *(29)*, FOXC2 (regulating lymphatic growth and valve formation and mutated in nearly all affected family members studied with lymphedema–distichiasis syndrome) *(30)* and SOX18 (mutated in hypotrichosis–telangiectasia–lymphedema syndrome) *(31)* have been identified. There are also at least 40 clinical syndromes involving prominent dysmorphologies of systems other than the lymphatic, which display combinations of multisystem anomalies particularly related to neural crest structures such as the face, brain, and eye and also the cardiovascular system and frequently associated with lymphedema and other lymphatic system abnormalities occasionally combined with venous anomalies *(32)*. Additional discoveries and clarification of the role of various lymphangiogenic

Table 1
Terminology Adjustment

Heme specific		*Lymph specific*
• Heme/Blood vasculogenesis	Vasculogenesis	• Lymphvasculogenesis
• Hemangiogenesis	Angiogenesis	• Lymphangiogenesis
• Hemangiotumorigenesis	Angiotumorigenesis	• Lymphangiotumorigenesis
• Tumor hemangiogenesis	Tumor angiogenesis	• Tumor lymphangiogenesis

factors and genes have resulted from examination of transgenic and mutant mouse models (e.g., VegfC deficient, Chy-1 and Chy-3, Angiopoietin knockout, Foxc2-deficient, and Prox1-deficient mice) *(29,33–40)*. This work taken together has confirmed that the VEGF and Angiopoietin families are crucial in lymphatic development and remodeling and the transcription factor, PROX1 and FOXC2 also plays some overarching influence.

Furthermore, it has become increasingly apparent that this molecular lymph specificity (Table 2) may not be as clear-cut as originally postulated based to some extent on information derived from isolated systems *(41–43)*. Overlap may occur, and normal developmental and a variety of pathologic states including cancer may alter specificity. Moreover, probably only a small proportion of the full array of lymphvascular

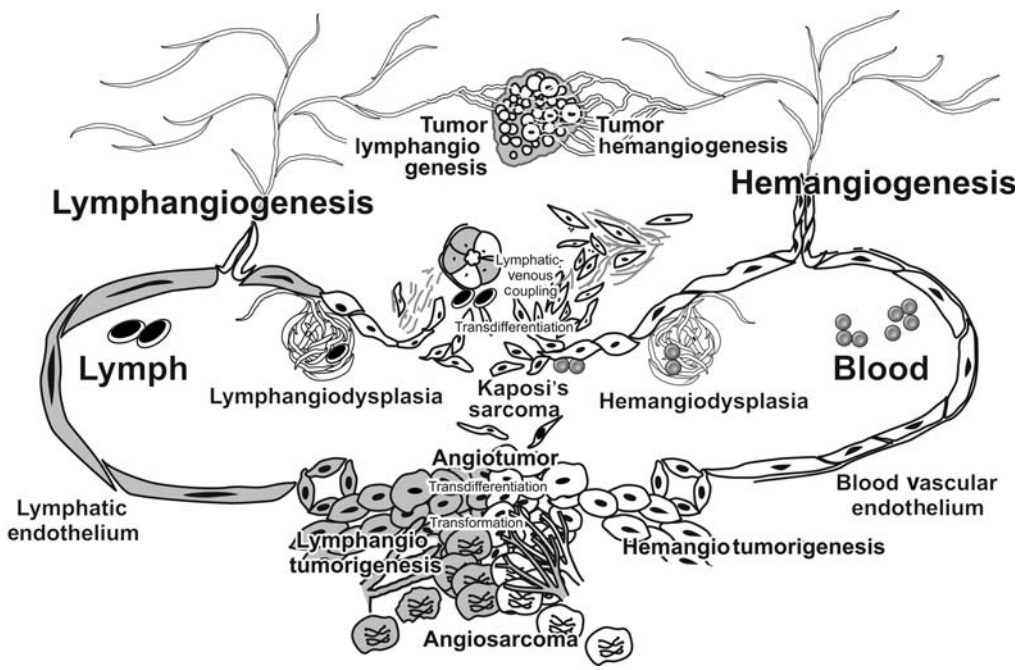

Fig. 5. Schematic representation of physiologic and pathologic processes common to and linking lymphatics with blood vessels and involving growth of lymphatic vessels (lymphangiogenesis) analogous to and often accompanied by growth of blood vessels (hemangiogenesis). Normally, these two vasculatures remain separate and connect directly only at a few strategic sites. In a variety of disorders, however, including tumor-associated angiogenesis, angiodysplasias, Kaposi sarcoma, and angiotumorigenesis including angiosarcoma, the two vasculatures come to resemble one another, interdigitate, and even merge indistinguishably (Reprinted/modified with permission from Witte MH, Witte CL, 1999).

Table 2
Vascular Endothelial Growth Factors and Receptors

	Vascular growth factors								
Receptors	*PIGF*	*VEGF-A*	*VEGF-B*	***VEGF-C****	***VEGF-D****	*VEGF-E*	***Ang1****	***Ang-2****	***Ephrin-B2****
VEGFR-1/Flt-1	♦	♦	♦						
VEGFR-2/Flt-1		♦		♦		♦			
VEGFR-3/Flt-4*				♦	♦				
Neuropilin-1	♦	♦	♦			♦			
Neuropilin-2*	♦	♦		♦					
Tie-1							♦		
Tie-2*							♦	♦	
EphB4									♦

♦ Corresponding growth factor-receptor combinations

* **Implicated specifically in lymphatic development *(bold and italic)*** in addition to transcription factors Prox1, FOXC2, Net, and SOX18.

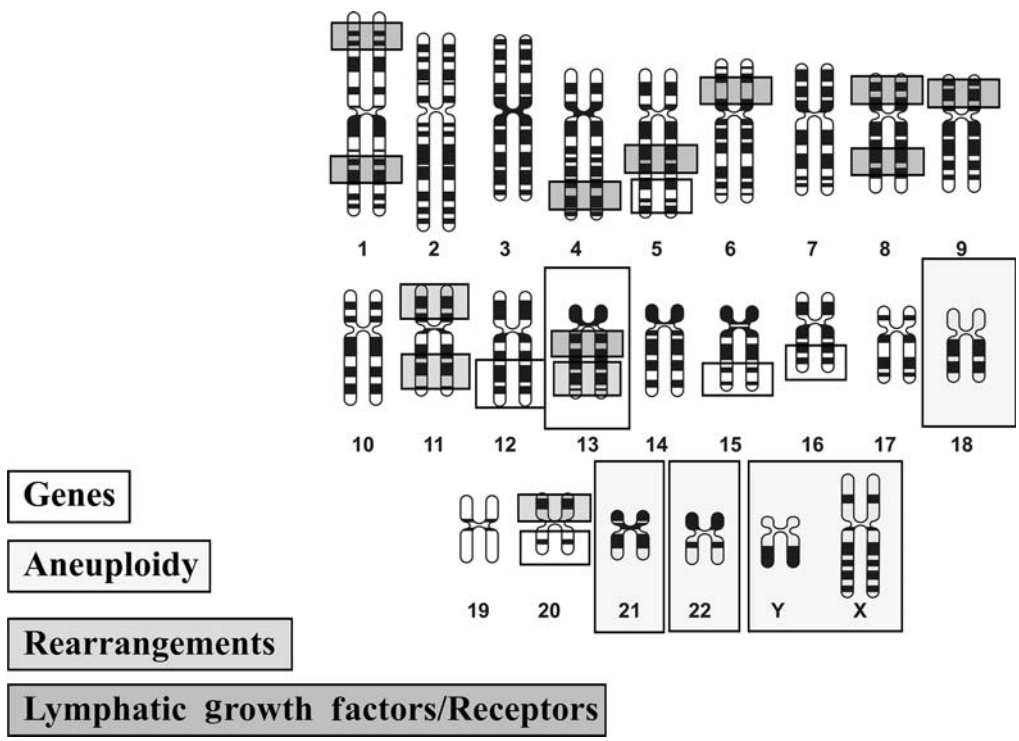

Fig. 6. Genomics of lymphedema-angiodysplasia syndromes displaying mutation of known genes for familial Milroy lymphedema subpopulation (VEGFR3 at chromosome 5 q34–35), lymphedema-distichiasis (FOXC2 at chromosome 16 q24), and hypotrichosis, lymphedema, telangiectasia (SOX18 at chromosome 20 q13), and linkage locations for Aagenaes syndrome (chromosome 15) and Noonan syndrome (chromosome 12). In addition, aneuploidies and rearrangements involving other chromosomes are associated with other lymphedema-angiodysplasia syndromes. If one adds the gene locations of key lymphatic growth factors, receptors, and transcription factors, almost all human chromosomes are potentially implicated. (Reprinted with permission from *(32)*).

angiogenic factors has been discovered and their interacting molecular/signaling pathways elucidated, providing a cautionary note to the rapid translation of molecular biologic insights and findings in transgenic mouse models from bench to bedside through novel lymphatic-specific therapeutics.

And once again questions that seemed to be definitively answered with molecular techniques are actually still open to controversy and debate including the centrifugal versus centripetal origin of the lymphatic system and the mechanisms of lymphatic remodeling.

6. ANGIODYSPLASIAS, HEM/LYMPHANGIOTUMORIGENESIS, AND TUMOR ANGIOGENESIS

Within the broad literal definition of "angiodysplasia" as an abnormal growth of vessels—too much, too little, malformed, and even neoplastic *(17,42–44)*—a bewildering array/constellation of poorly classified angiogenic, angioproliferative, and angiotumorigenic (benign/malignant) disorders (Table 3 and Fig. 5) continue to perplex clinicians in regard to differential diagnosis, course, aggressiveness, and treatment; only a few specific genes have been implicated. The cautionary note above is reemphasized in the context of clinical disorders—neoplastic and developmental—and included the reliability of heme/lymph-specific markers can be questioned.

A variety of tumors—both benign and malignant—arise from the blood and/or lymphatic vasculature, and their classification and treatment as well as the occurrence of spontaneous remission remain puzzling and challenging. Patient 1, the young lady who presented in 1982 with a rare and life-threatening massive, "disseminated" lymphangiotumor, raised many questions when we first encountered her *(10)* (Figs. 1 and 8): what is this entity—developmental disorder or neoplasm with distant spread to bone—embryonic rests (hamartomas) or true tumor that can invade, spread or originate in distant sites, even kill—but can also enter a prolonged resting stage as did her cultured cells after a few years and her tumors after massive debulking of the "primary" one? What led to its sudden growth and would it recur? Tools including distinctive lymphatic markers were not available to approach these questions 25 years ago. These questions—still largely unanswered with many more generated since—were and are fundamental to lymphatic endothelial biology, lymphangiogenesis in vitro and in vivo, and the fine line between developmental and neoplastic disorders. Diagnostic and therapeutic approaches as well as prognostication for the patient were (and remain) a formidable and frustrating challenge. Thus began our 25-year odyssey in lymphvascular biology from 1982 until the present day (vide infra).

Table 3.
Clinical Examples of Hemangio/Lymphangiodysplasias

A bewildering, poorly classified constellation of angiogenic, angioproliferative, angiotumorigenic (benign/malignant) disorders:
• Vascular "birthmarks"
• Capillary, cavernous, etc., hemangiomas, lymphangiomas and mixed angiomas
• Angiomatosis including "benign metastasizing" lymphangioma
• Histiocytoid hemangioma
• Malignant endovascular papillary angioendothelioma
• Epitheliod hemangioendothelioma(tosis)
• Kaposi's sarcoma and Kaposiforme
• (Lymph)angiosarcoma

After Patient 1, our Patients 2 and 3 *(17)* (Fig. 7) subsequently presented striking contrasts in hem/lymphangiotumorigenesis in vivo, the latter spontaneously resolving the lymphatic vascular component (contrary to common dicta about unlikely involution of lymphangiomas compared to hemangiomas), and the other exhibiting uncontrolled growth (despite "benign" histology) leading to the infant's rapid demise despite heroic efforts *(17)*.

And Kaposi's sarcoma (KS) (Figs. 5 and 8, right upper insert) also captured our interest (its nature, course, benign vs. malignant and its metastatic multifocal, undifferentiated, and transdifferentiated features) *(45,46)*. Does KS arise from lymphatic primordium (commonest theory), blood vascular cells, or stem cell/angioblasts? How are these stimulated by viral (HHV8) transformation and immunosuppression? Is KS analogous to tumor hem/lymphangiogenesis overlapping progressively to benign/malignant hem/lymphangiotumorigenesis?

Tumor angiogenesis, generally referring to hemangiogenesis, has been extensively studied since Folkman's original observations and also has become a major therapeutic target including by blockbuster drugs such as Genentech's Avastin. Tumor lymphangiogenesis *(47,48)*, a phenomenon whose very existence until recently was questioned, is now a "hot topic" for investigation and discussion. Yet, cells giving rise to the two vasculatures overlap and may

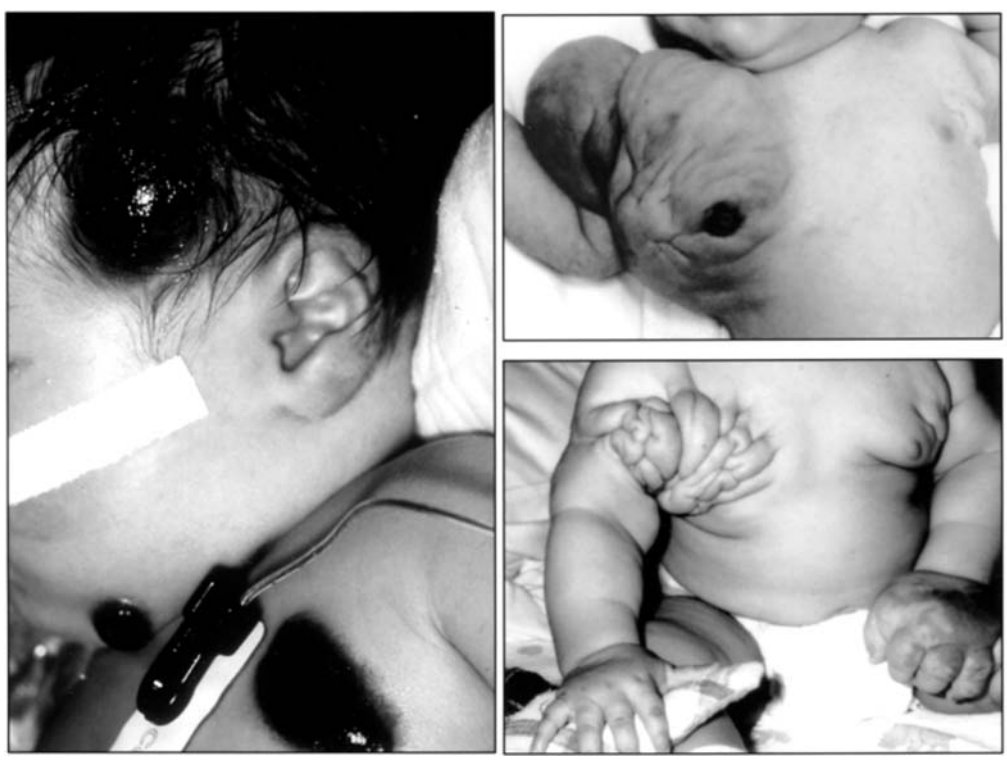

Fig. 7. *Left*, Patient 2: Neonate with rapidly fatal diffuse hemangiomatosis (exploding cutaneous hemangiomas shown here on the face and upper left chest and also replacing the liver) without histologic features of malignancy. *Upper right*, Patient 3: Female infant with diffuse mixed lymphangioma-hemangiomatosis syndrome involving the right chest wall, upper arm, shoulder girdle, and left hand (not shown). Four months later (*lower right*), the chest wall/shoulder lesion had largely involuted spontaneously whereas the painful hemangiomatous lesions on the left hand and forearm were unchanged. (see text for further details). (Modified with permission from Witte MH, Witte CL, 1999).

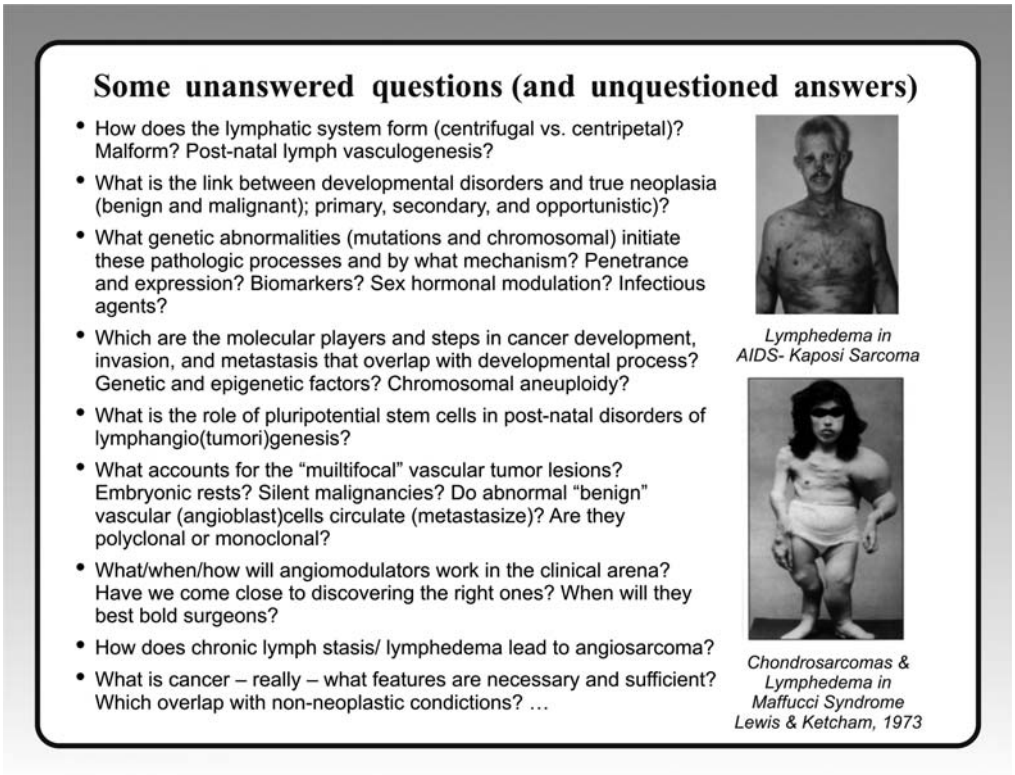

Fig. 8. Some unanswered questions (and unquestioned answers).

interchange in specific pathologic settings (Fig. 5) and come to resemble each other in different regional settings. At the present time, a variety of companies, stimulated by thought leaders in the angiogenesis field, are vigorously pursuing pharmaceuticals with antilymphangiogenic properties and testing these in preclinical models for application to patients with cancer (presentations at this conference).

7. ANGIOMODULATION AND ANGIOPROTECTION

At the 18th International Congress of Lymphology (1997) in Madrid, we presented a conceptual scheme (Fig. 9) to approach understanding of these processes—under normal and pathologic conditions *(49)*. This scheme was intended as a framework for research and therapeutic approaches with angiostimulators and angioinhibitors—specific to lymphatic or blood vasculature or directed at both. A few specific molecular targets had been identified at that time, but a variety of earlier less-specific agents—some already FDA approved for other conditions *(50)*—were known to have angiomodulatory effects (and have recently entered clinical trials) *(17,49)*. During the past decade and particularly the past 5 years a wide array of angiomodulators—including highly specific molecular based ones—have been proposed with different mechanisms of action. Some (hem)angiomodulators have entered clinical trials in coronary artery disease and cancer (with some success in the latter with Genetech's Avastin). Others specifically target blood vessels or lymphatics through their relatively

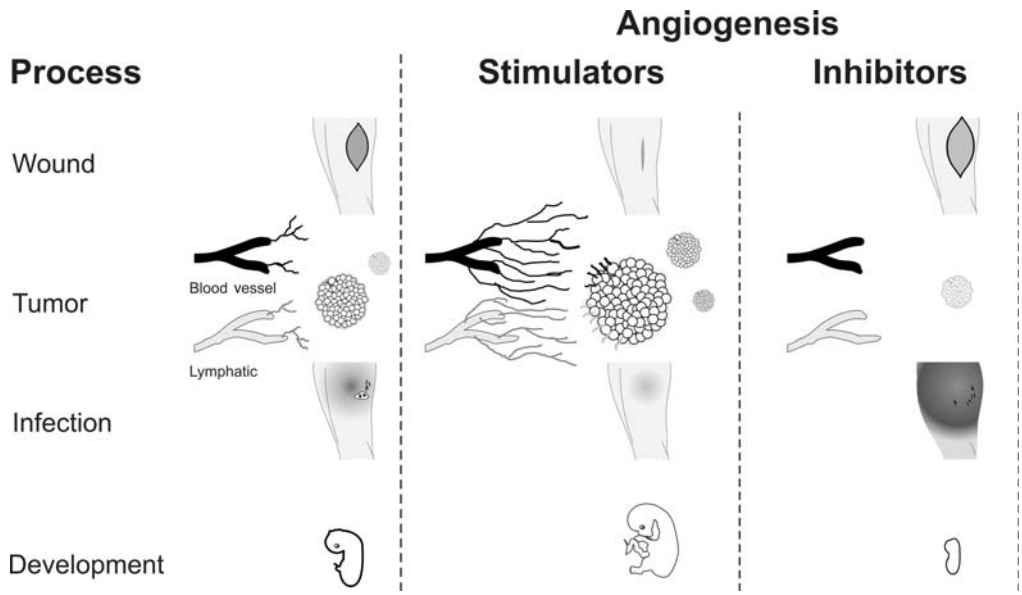

Fig. 9. Schematic representation of examples of angiostimulation and angioinhibition involving both lymphatic (lymphangiogenesis) and blood (hemangiogenesis) vasculature and the known or likely participation in common biological processes. [Reprinted with permission from *(49)*].

selective receptors. Thus, targeting one or the other but not both vasculatures now is clinically relevant, and specificity may become a limiting factor in clinical effectiveness and side effects.

8. UNANSWERED QUESTIONS AND UNQUESTIONED ANSWERS (CONTINUED) (FIG. 8)

In 2007, Patient 1 *(10)* (Fig. 1)—long lost to follow-up—made contact with us again after a hiatus of 25 years. She had done well postoperatively without recurrence of the "primary" cervico-mediastinal lymphangioma. Scattered bony lymphangiomas persisted but had not enlarged, and she now gave a history suggestive of familial occurrence of her disease, a variant of Gorham's syndrome or "vanishing bone disease" (yet bone is supposed to lack lymphatics), not currently listed as hereditary in the OMIM database. What had caused her lymphangioma/cystic hygroma to grow so dramatically back then and, after bold massive resection, to enter a prolonged latent period with stable lesions? And why had the lymphatic endothelium derived from her lymphangioma cells remained in culture, spontaneously forming tubes for several years but then entered dormancy as did questions about her? And other questions: How long will it be before lymphangioinhibitor or tailored gene therapy is preferable to, or can it even match, the long-term remission secured by the courageous surgeon who accepted a desperate patient and debulked whatever was debulkable, and with fingers crossed, safely and permanently excised and halted the lymphangiostimulatory/tumorigenic process?

What accounts for the similarities and differences between blood and lymphatic vessels and the boundaries and transdifferentiation between the two? How can they be manipulated for disease regression and control or even prevention? And how long will it take and, at what costs, for molecular medicine, while intellectually satisfying, to surpass the results of advancing

conventional medicine and surgery. "Benign metastasizing lymphangioma"—"multifocal embryonic rests," what do these terms really mean and what are the proximate mechanisms of lymphogenous and hematogenous spread? Can and do cells from benign tumors (e.g., lymphangioma) and even nontumors (e.g., Whipple cells *(51)*) circulate and relocate (metastasize) and under what conditions and which molecular mechanisms? When and how do the lymphatic and blood vasculatures overlap or develop in different directions but in some disturbances may meet and merge again, and in cancer may further synergize, compete, and overlap *(48)* (Fig. 5).

9. CONTINUING SAGA AND CONTROVERSY ABOUT THE LYMPHATIC SYSTEM

"Sembra una fatalita, che il sistema linfatico abbia in ogni tempo suscitado le piu violente discussione" (It seems inevitable that the lymphatic system has at all times provoked the most vigorous/even violent discussion.)—Milan journalist, 1853 [cited in *(16)*].

And so, the saga continues as controversy mingled with fascination surrounds the lymphatic system—its investigators (old and new) and its origins; similarities, differences, and complex relationships with the blood vasculature; and participation in both rare and common developmental and neoplastic disorders. Whatever direction the path of discovery may take, research and discussion should be informed by the precise and long overdue terminology adjustment recommended here until a more accurate one based on improved understanding comes along to replace it.

ACKNOWLEDGMENT

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9 Tumor Lymphangiogenesis: What We Know and Don't Know

Antje Neeb, PhD and Jonathan P. Sleeman, PhD

Contents

ABSTRACT

The lymphatic vasculature represents a major conduit through which tumor cell metastasize. In addition to structural considerations and passive mechanisms that facilitate entry of tumor cells into lymphatic capillaries, a number of processes have been discovered whereby tumor cells actively promote their entry into the lymphatics. One of these processes is tumor-induced lymphangiogenesis. Activation of VEGFR-3 on lymphatic endothelial cells (LECs) by its ligands VEGF-C and/or VEGF-D produced by tumors is the best-studied regulator of tumor-induced lymphangiogenesis. However, a number of other pro-lymphangiogenic factors operative within tumors have additionally been discovered. Progress is being made in understanding the signal transduction pathways and their end points that orchestrate lymphangiogenesis. Together, these findings are supporting attempts to therapeutically interfere with tumor-induced lymphangiogenesis. Nevertheless, many outstanding issues remain to be addressed, including possible side effects of such therapeutic interference. Furthermore, additional active mechanisms that

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promote the formation of lymph node metastasis are emerging, including chemokine-mediated chemotaxis and remote tumor-induced changes in the lymph node microenvironment that support subsequent metastasis formation. The relative contribution of these different mechanisms to the process of metastasis remains to be investigated.

Key Words: metastasis; lymphangiogenesis; lymph node; VEGFR-3; VEGF-C; VEGF-D

1. INTRODUCTION

It has long been recognized that metastasis of cancer cells through the conduit provided by the lymphatic vasculature has a central role in the dissemination of tumors *(1)*. Metastasis to the regional lymph nodes is often the first sign that the tumor has progressed to metastatic competence *(2–5)*, and regional lymph node status is highly clinically significant for the prognostic evaluation of a broad range of tumor types *(6)*. Regional lymph node metastases form preferentially despite the fact that tumor cells can be detected in the blood stream early on after the primary tumor develops *(7)*. Furthermore, histological analysis of the sentinel lymph node, the first lymph node receiving lymph flow from the primary tumor *(8)*, is now widely used to determine whether metastatic spread has occurred and whether metastases at distant sites are likely *(9)*. Follow-up analyses of patients receiving sentinel lymphadenectomies allow the estimate to be made that only 20% of systemic metastases are derived from tumors that bypass the lymphatic route *(2)*.

A variety of passive mechanisms contribute to the entry of invasive tumor cells into the lymphatics. The high internal pressure within the tumor produces a constant flow of interstitial fluid away from the tumor toward the draining lymphatics *(1,10)* that can carry detached tumor cells. End-stage lymphatic capillaries themselves offer relatively low resistance to the entry of tumor cells, as the lymphatic endothelium has loose intercellular junctions and is encompassed by no or only an incomplete basement membrane (reviewed in *[11,12]*). The flow of the lymphatic fluid in end-stage lymphatic capillaries is passive, meaning that tumor cells trafficking via the lymphatics do not experience the same shear forces and pressure-mediated stress as tumor cells in the blood circulation, enhancing their survival.

In addition to mechano-physical mechanisms such as those outlined above, it has become increasingly clear over the last years that tumors actively interact with the lymphatic vasculature and thereby facilitate their entry into the circulatory system. A particular focus of research in this area has been on lymphangiogenesis, the formation of new lymphatic vessels, a process that is precisely governed by a complex network of growth factors, cytokines, and chemokines *(13,14)*. A large body of literature now indicates that by producing pro-lymphangiogenic factors, tumors are able to induce lymphangiogenesis. The resulting increase in lymphatic vessel density (LVD) is thought to increase the likelihood that invasive tumor cells enter the lymphatics, and thereby contribute actively to tumor metastasis. In this review, we summarize the current knowledge base in this area, place tumor-induced lymphangiogenesis within the context of other newly discovered mechanisms that may also actively promote metastasis via the lymphatics, and identify areas where further research is warranted.

2. CANCER DIAGNOSIS AND PROGNOSIS: HOW IMPORTANT IS THE ROLE OF THE LYMPHATICS?

A number of studies have examined increased LVD in and around primary tumors and have investigated its relationship with lymph node status and/or survival (reviewed in *[15]*). For most cancer types, there is a tendency for LVD to correlate with lymph node metastasis, although

contradictory data exist for pancreatic, breast and prostate carcinomas. For melanoma, breast and head and neck squamous cell carcinoma (HNSCC) a correlation between high LVD and poor survival has been reported, although no such correlation was found for other carcinoma types. Tumor-associated lymphatic vessels have been reported to be both intratumoral and peritumoral. The extent to which these vessels represent lymphatics that have been coopted by tumors, or whether their existence is due to lymphangiogenesis is generally not addressed in these studies, although proliferative activity of tumor-associated lymphatics has occasionally been examined and found, supporting the notion that lymphangiogenesis contributes to the tumor-associated lymphatics in human tumors (e.g., *[16,17]*).

While some of these correlative studies lend credence to the notion that enhanced LVD caused at least in part by tumor-induced lymphangiogenesis can correlate with lymph node metastasis formation and poor survival, this is by no means always the case. Several factors may account for this. A higher density of lymphatic vessels in the vicinity of primary tumors may be necessary but not sufficient for metastasis, as the tumor cells may not have acquired properties required for metastasis, such as invasiveness. Conversely, depending on the location of the primary tumor, the density of lymphatic vessels may be sufficient to support metastasis to lymph nodes without the need for lymphangiogenesis. Thus, depending on the cohort of patients selected, a direct correlation between LVD and lymph node metastasis or survival may not be observed.

3. PROLYMPHANGIOGENIC FACTORS: WHAT IS THEIR RELEVANCE IN TUMOR-INDUCED LYMPHANGIOGENESIS?

The most widely investigated receptor–ligand pair involved in tumor-induced lymphangiogenesis is the vascular endothelial growth factor receptor family member VEGFR-3. VEGFR-3 is a transmembrane receptor tyrosine kinase that is expressed on the surface of lymphatic endothelial cells (LECs) and is activated by VEGF-C and VEGF-D, members of the vascular endothelial growth factor family *(18)*. The involvement of this receptor–ligand pair in regulating tumor-induced lymphangiogenesis has been extensively documented, both in the context of correlative studies that have examined expression of these factors in a variety of human tumors and in functional studies using animal models. This body of literature has been extensively reviewed (e.g., *[15,19–21]*) and will not be further expanded upon here.

Another member of the vascular endothelial growth factor receptor family VEGFR-2 has also been implicated in regulating tumor-induced lymphangiogenesis *(22,23)*. VEGFR-2 is expressed on collecting lymphatic vessels and capillaries undergoing lymphangiogenesis (*[24,25]*; reviewed in *[18,26]*). In addition to being activated by VEGF-A, the fully processed forms of VEGF-C and VEGF-D can also bind to and activate VEGFR-2. VEGF-A isoforms have been implicated in the induction of lymphatic hyperplasia *(27,28)*. VEGF-D induces the formation of heterodimers between VEGFR-2 and VEGFR-3 *(29)*, and in vitro studies have demonstrated that selective activation of either VEGF-2 or VEGF-3 induces proliferation of cultured LECs *(24,25,30)*. Other studies indicate a role for VEGFR-2 in enlargement of vessel diameter, but not in sprouting lymphangiogenesis *(31)*, and suggest a cooperative interaction between VEGFR-2 and VEGFR-3 for LEC migration and proliferation *(32)*. In this regard, it is interesting to note that VEGFR-2 activation was found not to be sufficient for the generation of new lymphatic vessels, and that furthermore it was not able to rescue lymphatic regression induced by blocking VEGF-C and VEGF-D *(31)*. The picture that is emerging is that VEGFR-2 activation may be a modifier but not necessarily an initiator of lymphangiogenesis. This may

possibly explain why many tumors do not exhibit tumor-induced lymphangiogenesis despite producing VEGF-A. Clearly, more studies are required to elucidate the relative importance and mechanism of the contribution of VEGFR-2 to tumor-induced lymphangiogenesis.

In addition to binding VEGFR-2 and VEGFR-3, VEGF-C/D has also been reported to bind α9β1 integrins *(33)*. The α9β1 integrin isoform is detectable on LECs but not on blood endothelial cells (BECs) *(34)* and targeted deletion mutants exhibit severe defects in the formation of lymphatic vessel structure *(35)*, strongly indicating that the interaction between VEGF-C/D and α9β1 integrins is relevant and necessary for proper vessel development. The α9β1 integrin may also play a role in regulating VEGF-A activity, as it has recently been reported that it binds directly to VEGF-A *(36)*. Furthermore, VEFG-C/D additionally binds to the VEGFR coreceptor Neuropilin-2, a semaphorin receptor that is also expressed on LECs *(37)*. Neuropilin-2-deficient mice succeed in building a lymphatic system but show a reduced number of small lymph vessels and severe hyperplasia of the lymph capillaries from E13 to birth *(38)* assigning more of an enhancer function than an essential role in lymphangiogenesis to Neuropillin-2. A role for α9β1 integrin and neuropilin-2 in tumor-induced lymphangiogenesis remains to be determined.

A number of other growth factors and cytokines in addition to members of the vascular endothelial growth factor family have been shown to induce lymphangiogenesis (reviewed

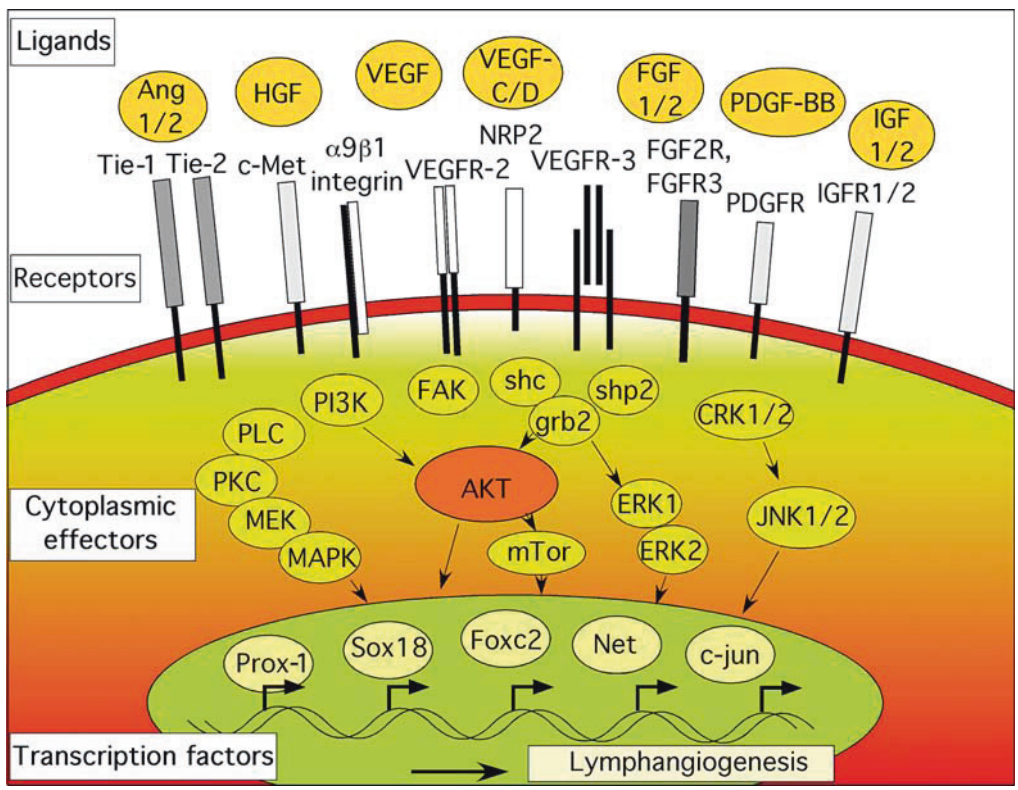

Fig. 1. Signaling molecules possibly involved in tumor-induced lymphangiogenesis. In this figure, several growth factor and cytokine receptors and their ligands are depicted that have been implicated in lymphangiogenesis and are expressed in the context of tumors. Examples of signal transduction pathways that are addressed by some of these receptors and that have been shown to be effectors in the process of lymphangiogenesis are shown. Finally, transcription factors that are targeted by these signals transduction pathways and/or have been shown to play a role in aspects of lymphangiogenesis are depicted. (*see* Color Plate 6)

in *[39]*; see Fig. 1 and Color Plate 6). The Tie receptor tyrosine kinase family consists of two members Tie-1 and Tie-2. These receptors and their ligands angiopoietin-1 (Ang-1) and Ang-2 play a role in the development of the lymphatics *(40)*. Cross-regulatory loops exist between the VEGFR and the Tie receptors, as Ang-1 expression in the adult mouse skin induces the formation of lymph vessels accompanied by VEGFR-3 upregulation in LECs *(18)* and VEGF-C induces Ang-2 expression in cultured LEC *(30)*. Other receptors whose activation has been shown to promote lymphangiogenesis include the hepatocyte growth factor receptor (c-Met *[41]*), EphrinB2 *(42)*, platelet-derived growth factor receptor (PDGFR-αβ *[43]*), lymphotoxin beta receptor *(44)* and receptors for insulin-like growth factors 1 and 2, and members of the fibroblast growth factor family *(45–47)*. Several of these receptors and their ligands are known to be expressed in the context of tumors, yet for most a role in tumor-induced lymphangiogenesis remains to be demonstrated. PDGF-BB has been shown to induce lymphangiogenesis and lymphatic metastasis in animal models, and HGF might also contribute via indirect mechanisms *(48,49)*. Ang-2 expression correlates with lymph node metastasis and poor survival in breast cancer *(50)*.

4. SIGNAL TRANSDUCTION PATHWAYS THAT MEDIATE LYMPHANGIOGENESIS: COMMON MOTIFS?

The process of lymphangiogenesis requires the orchestration of many complex cellular processes such as proliferation, migration, invasion, and tubule formation. We are only just beginning to understand the signal transduction pathways and transcription factors that coordinate these processes. Activation of VEGFR-3 by its ligands VEGF-C or VEGF-D results in transphosphorylation of tyrosine residues in the cytoplasmic portion of the dimerized receptor, mediated by the intracellular kinase domains *(51)*. The signal transduction pathways that are thereby activated are still incompletely defined. In cultured LECs, activation of VEGFR-3 results in protein kinase C-dependent activation of the p42/∼p44 MAPK signaling cascade and induction of Akt phosphorylation, protecting the cells from apoptosis and inducing proliferation and migration *(29)*. More recent work has dissected how VEGFR-3 signals via the ERK, JNK, and AKT pathways *(52)*. Phosphorylation of VEGFR-3 tyrosine 1,063 recruits CRKI/II to the receptor, inducing c-jun expression via JNK1/2. Phosphorylation of tyrosine residues 1,230/1,231 recruits GRB2, activating in turn ERK1/2 and AKT.

Lymphangiogenic signaling via FGFR-2 involves signaling via the Akt/mTOR/p70S6 kinase pathway *(53)*. The common activation of the AKT pathway by both VEGFR-3 and FGFR-2 provides first evidence for similarities between the signal transduction pathways activated in LEC by prolymphangiogenic signaling. Furthermore, in this context it is interesting to note that rapamycin, a specific inhibitor of mTOR, is able to inhibit tumor-induced lymphangiogenesis and lymphatic metastasis *(54)*. Although in this study the effect on tumor-induced lymphangiogenesis and lymphatic metastasis was attributed to reduced transcription of VEGF-C, direct effects on receptor-mediated prolymphangiogenic signaling may also be involved.

Genetic manipulation in mice has allowed the identification of additional signal transduction components that regulate lymphangiogenesis. Sprouty/Spred family proteins, negative regulators for growth factor- and cytokine-induced RAS–ERK activation, have recently been implicated in the regulation of lymphangiogenesis. Using *Spred-1*/*Spred-2* double-knockout mice, it has been demonstrated that Spreds are key regulators of embryonic lymphangiogenesis and that they can specifically regulate VEGF-C signaling by suppressing VEGFR-3-mediated ERK and Akt activation *(55)*.

Transcriptional activation is one of the end points of signal transduction pathways that are activated in response to prolymphangiogenic factors. VEGF-C, for example, regulates expression of a number of genes *(56)*. Targeted deletion in mice of a number of transcription factors

including Foxc-2, Elk3 (Net), Prox-1, and Sox18 *(18)* results in a lymphatic phenotype. The extent to which these transcription factors are involved in determining lymphatic characteristics compared to being targets for prolymphangiogenesis signaling remains to be clarified. For example, the homeobox transcription factor Prox-1 plays an important role in regulating the expression of genes that determine aspects of LEC morphology and behavior *(57)*. However, how Prox-1 is wired into the regulatory pathways that orchestrate lymphangiogenesis, what other transcriptional regulators play a role, and how different aspects of lymphangiogenesis (e.g., sprouting lymphangiogenesis compared to capillary enlargement) are regulated at the genetic level is not known. Other end points of prolymphangiogenic signal transduction pathways are likely to include the cytoskeleton and adhesion complexes amongst others, but these remain to be identified.

5. OTHER MECHANISMS THAT ACTIVELY PROMOTE TUMOR ENTRY INTO THE LYMPHATICS: WHAT IS THE RELATIVE IMPORTANCE OF TUMOR-INDUCED LYMPHANGIOGENESIS?

Tumor-induced enrichment of LVD in and around the tumor is not the only manner in which the tumor interacts with and influences the lymphatics to promote metastasis, as described below (see Fig. 2 and Color Plate 7). The relative importance of these different mechanisms for the formation of lymph node metastasis remains to be deciphered.

5.1. Chemokines

Lymph node metastasis formation correlates with the expression of chemokine receptors on tumor cells, in particular CCR7 and CXCR4 *(58–60)*. CCR7 plays a role in guiding CD4-positive memory T cells and dendritic cells into the lymphatics. CCL19 and CCL21, the ligands of CCR7, are involved in T-cell and dendritic cell trafficking to the lymph nodes. Interestingly, expression of CCL19 and CCL21 correlates with lymph node metastasis in breast cancer, HNSCC, lung cancer, and gastritic carcinoma *(61–63)*. CCL21 is expressed by LEC *(64–66)*. Recent studies indicate a functional role for CCR7-mediated chemotaxis in lymph node metastasis formation *(67)*. Experimental overexpression of CCR7 in B16 mouse melanoma cells enhanced the incidence of lymph node metastasis in mice. This effect was completely blockable by anti-CCL21 antibodies. This migration could be selectively blocked by anti-CCL21 antibodies but not by interference with VEGFR-3 signaling *(65,66)*. The observation in vivo that CCR7-positive tumor cells grow toward a depot of LECs supports the hypothesis that tumor cells are chemotactically attracted to regions of high LEC density due to secretion by LEC of chemokines such as CCL21 *(65,66)*. In addition to this paracrine effect, recent work shows that CCR7-positive tumor cells can themselves produce CCR7 ligands. Interstitial fluid flow around these cells creates a stronger concentration of autocrinely produced CCR7 ligand on the leeward side of the interstitial fluid flow, creating a chemotactic gradient that guides tumor cell migration in the direction of intestinal flow. Together, these data suggest that CCR7 ligand secretion by LECs and/or tumor cells can promote migration toward the draining lymph node.

5.2. Vascular Mimicry

It has recently emerged that intratumoral lymphatic vessels may be formed at least in part by vascular mimicry or transdifferentiation. Several studies have shown that circulating CD11b-positive, LYVE1-positive macrophages are able to integrate into lymphatic vessels and to form lumen-containing capillaries *(68,69)*. This has also been reported for tumor-associated lymphatics *(70)*. The relative contribution of these macrophage-derived cells to the lymphatic

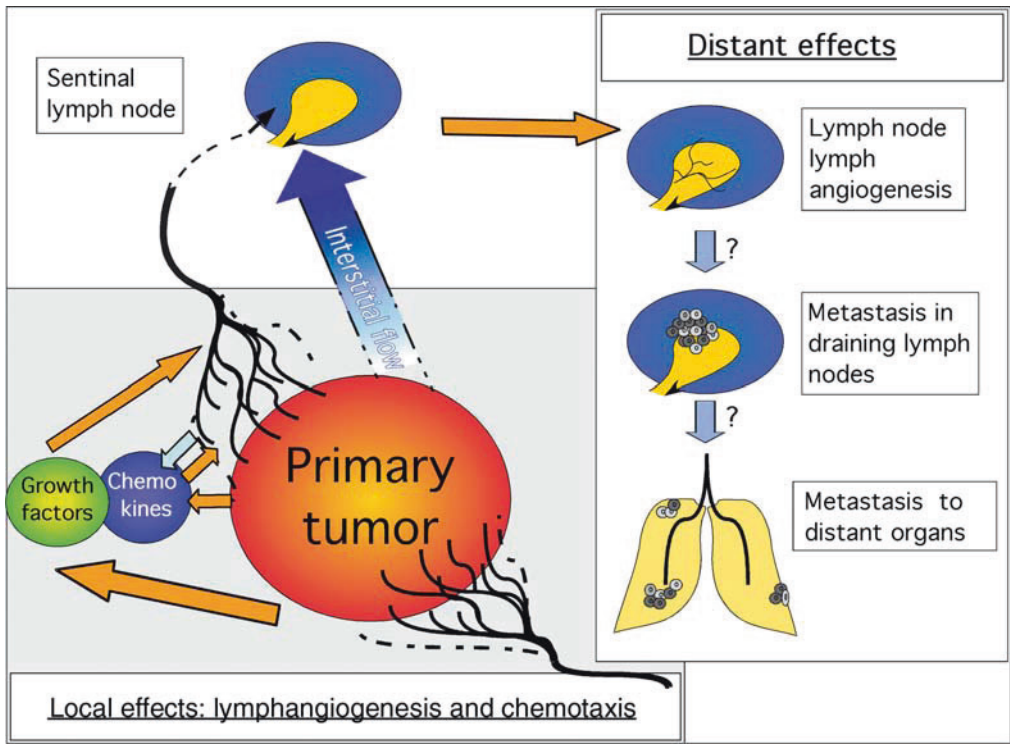

Fig. 2. Tumor–vessel interactions: local and distant effects. Primary tumors secrete growth factors that can act locally on the lymphatics to increase vessel diameter and to increase the number of vessels. Tumors can also produce chemokines, and autologous chemotactic gradients can be generated around tumor cells by these factors as a consequence of interstitial fluid flow. These gradients guide tumor cells to the lymphatic vessels. In addition, chemokines produced by the lymphatics can also attract tumor cells. More distantly, tumor-derived factors can also induce lymphangiogenesis in draining lymph nodes, which serves to increase interstitial fluid flow to the lymph nodes and may also be a prerequisite for the formation of lymph node metastases. Lymph node metastases themselves may also contribute to the formation of metastases in distant organs. (*see* Color Plate 7)

vasculature in the context of tumors, the regulation of their recruitment into the lymphatic capillaries, and their importance for metastasis via the lymphatics remains to be demonstrated. Initial observations with animal models suggest that lymphangiogenesis from preexisting lymphatic vessels accounts for most of the tumor-associated lymphatics *(71)*, while studies with human melanomas suggest that the majority of intratumoral lymphatics in melanomas may be derived from macrophage precursors *(70)*.

5.3. Lymph Node Lymphangiogenesis

The term "premetastatic niche" *(72,73)* describes tumor-induced premetastatic changes that support the formation of metastases in the microenvironment of organs to which tumors will subsequently metastasize. Recent work suggests that tumors may induce the formation of premetastatic niches in lymph nodes prior to lymph node metastasis formation, as tumors appear not only to induce lymphangiogenesis in their immediate vicinity but also distally. Hirakawa et al. *(74)* have reported that VEGF-C-expressing tumors induce lymphangiogenesis in sentinel lymph nodes in addition to the local tumor environment. Sentinel lymph node lymphangiogenesis occurs before metastatic cells enter the lymph nodes and is further augmented once metastases form. Similar findings have been made for VEGF-A-overexpressing primary skin tumors in

mice, which were found to induce lymphangiogenesis in the sentinel lymph nodes before metastatic tumor cells arrived *(28)*. These findings have been supported by analysis of a syngenic mouse melanoma model, where footpad injection of B16 melanoma cells did not result in local lymphangiogenesis but led to early extensive growth of lymphatic vessels in popliteal lymph nodes before metastatic tumor cells were detectable in the lymph nodes *(75)*. Similar observations have been made in human breast tumors, in which lymphangiogenesis was observed in 25% of noninvolved axillary lymph nodes *(76)*. Tumor-induced sentinel lymph node lymphangiogenesis substantially increases lymph flow to the lymph node *(77)*, and by increasing flow through the tumor-draining lymph nodes may actively promote metastasis via the lymphatics. However, tumor-induced sentinel lymph node lymphangiogenesis may also prepare lymph nodes for receipt of metastatic tumor cells by inducing a premetastatic niche microenvironment that is permissive for growth of metastases *(20)*.

6. MANIPULATION OF TUMOR-ASSOCIATED LYMPHATICS: THERAPEUTIC APPLICATIONS?

The observation in animal models that inhibition of tumor-induced lymphangiogenesis is sufficient to reduce the incidence of metastasis has obvious therapeutic applications. As an example, several studies have sought to inhibit VEGF-3-mediated lymphangiogenesis. One approach has been the production of antibodies that either block the activity of the ligands VEGF-C and VEGF-D *(56,78)* or prevent their binding to VEGF-3 via occupation of the binding domain on the receptor *(79,80)*. In addition, soluble dimeric fusion proteins containing the extracellular binding site of VEGFR-3 have proven to be very effective in nonproductively sequestering VEGF-C/D, thereby reducing the activation of VEGFR-3 and suppressing lymphangiogenesis *(71,81,82)*. Small molecular weight inhibitors that interfere with the signaling of VEGFR-3, for example, by inhibiting its kinase activity, have been developed in our group and others *(82–86)*.

Despite recent excitement concerning therapeutic application in the arena of cancer therapy, there are a number of outstanding questions to be resolved regarding the regulation of tumor-induced lymphangiogenesis, and its relative contribution to the process of metastasis. With the recognition that a number of different growth factors have the capacity to induce lymphangiogenesis, their relative contribution to tumor-induced lymphangiogenesis needs to be assessed: blocking only one prolymphangiogenic signal may not be sufficient to suppress tumor-induced lymphangiogenesis. Furthermore, it will be important to identify which intracellular signaling pathways the various prolymphangiogenic factors activate in order to exert their prolymphangiogenic effect. By studying the interaction and networking of the pathways that are activated by the different prolymphangiogenic factors, it may be possible to identify regulatory nodes that could be therapeutically targeted in order to block the effects of multiple prolymphangiogenic factors. Moreover, attention to date has been focused fairly exclusively on prolymphangiogenic signaling. Negative counter-regulation may exist, activation of which may also prove effective in blocking tumor-induced lymphangiogenesis. Finally, the efficacy of targeting lymphangiogenesis in human tumors needs to be carefully assessed, given that it is emerging that tumor-induced lymphangiogenesis is only one of the several mechanisms that promote metastasis to lymph nodes and beyond.

Possible unwanted side effects of targeting of lymphangiogenesis for cancer therapy have to be considered. Lymphangiogenesis is induced after wounding, but newly formed lymphatic vessels regress as the wound resolves *(87)*. The significance of this transient induction remains poorly understood, and it remains to be fully investigated whether inhibition of lymphangiogenesis

could result in edema in regenerating tissue. Other side effects are likely to be dependent on the prolymphangiogenic pathway that is targeted. For example, VEGFR-3 is expressed on certain macrophages and is also found on monocytes in the cornea and in the blood *(88–91)*. These observations are consistent with a role for VEGFR-3 in the hematopoietic system, as indicated by the fact that VEGFR-3-deficient mouse embryos suffer from a 50% reduction in hematopoietic cell number without alteration in the number of hematopoietic stem cells *(92)*. In the brain VEGFR-3 seems to be essential for the proliferation of nestin-positive neuronal progenitors as well as oligodendrocyte precursors *(93)*. Thus, consequences of anti-VEGFR-3 therapies on these additional physiological functions of the receptor have to be considered. Similar arguments hold true for other tumor-associated prolymphangiogenic signaling mechanisms.

7. CONCLUSIONS

Tumor-induced lymphangiogenesis has emerged in recent years as a mechanism that can promote metastasis to lymph nodes, and may support further metastasis to additional organs. Targeting tumor-induced lymphangiogenesis may therefore find therapeutic application in the management of cancer. However, several prolymphangiogenic factors may be operative within a given tumor, meaning that multiple pathways may need to be targeted. Furthermore, it is becoming clear that additional mechanisms also exist that can support the development of lymph node metastases independently of tumor-induced lymphangiogenesis. The challenge now is to understand the relative importance of these factors and mechanisms to the process of metastasis to aid the design of appropriate experimental strategies.

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IV Diagnostic Imaging of Cancer

10 Molecular Imaging of Cancer: Receptors, Angiogenesis, and Gene Expression

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CONTENTS

ABSTRACT

There is an increasing desire to understand molecular mechanisms that regulate cancer growth and metastasis. Positron emission tomography (PET) imaging can be used for this purpose in research and clinical trials. An ever-growing number of PET tracers are now available to image biochemical alterations characteristic of the cancer cell or tumor-induced changes in the surrounding stroma. This chapter addresses angiogenesis, receptor expression, and gene imaging. Angiogenesis can be imaged using the compound ^{18}F arginine–glycine–aspartic acid (RGD) peptide, which binds specifically to $\alpha\nu\beta3$ integrin receptors expressed at the surface of proliferating endothelial cells. A number of receptors are critical for cancer development and progression. Presence and functional activity of receptors can be studied with PET probes, among which FDHT and FES are in clinical trials for imaging of the androgen and estrogen receptors, respectively. Gene expression can be imaged, and this will be of increasing importance as part of future clinical trials with stem cells or modified T cells, allowing for cell tracking in the human body and visualization of the degree and location of expression of a therapeutic gene.

Key Words: positron emission tomography; cancer; angiogenesis; receptor imaging

There is an increasing desire to understand molecular mechanisms that regulate cancer growth and metastasis as well as the interaction between the cancer cell and the surrounding stroma *(1,2)*. Positron emission tomography (PET), an imaging technology that uses the compounds labeled with positron emitting radioisotopes to image and measure biochemical processes *(3)*, can be used for this purpose in the research arena and as part of clinical trials. Clinically, more than 90% of PET studies are done using the compound ^{18}F fluorodeoxyglucose (FDG), which

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traces the Warburg effect, that is, a biochemical hallmark of cancer *(4)*. The biochemical rationale for FDG PET imaging was explained in detail as part of a prior paper *(5)*. However, many other PET radiotracers are available to image biochemical alteration characteristic for the cancer cell or the response in the surrounding stroma. Some of these principles and compounds are discussed as part of this presentation.

1. ANGIOGENESIS

Angiogenesis is necessary for cancer to grow beyond a certain size and is a general characteristic of any malignancy. Indeed, the generation of a lethal tumor mass requires tumor cell proliferation plus angiogenesis *(6)*. Tumor cell proliferation alone, in the absence of angiogenesis, may give rise to dormant, microscopic tumors of $\sim$1 mm^3 or less, but these in situ cancers are harmless to the host *(7,8)*. Further tumor growth can only occur after the "angiogenic switch" is turned on. This can be shown exemplary in an animal experiment *(9)*. Two groups of mice were inoculated with liposarcoma cells. In group A, inoculated with angiogenic liposarcoma cells, tumors grew rapidly to a lethal size after about 30–40 days. In another group of animals (B), inoculated with nonangiogenic liposarcoma cells, small tumors developed but remained microscopic (dormant) for up to one-third of the normal mouse life span. Tumors in both groups of animals showed similar capacity to excrete the proangiogenic factor VEGF-1, but cells in group B also synthesized and excreted large amounts of angiogenesis inhibitors (such as thrombospondin-1, TSP-1, or tissue inhibitor of metalloproteinase-1, TIMP-1). The development of tumor vessels in group B was therefore impaired. Nevertheless, these small tumors remained fully viable and showed rapid expansion after a switch to the "angiogenic phenotype," characterized by a shift in balance between proangiogenic and antiangiogenic factors. It is currently unclear what exact molecular mechanism(s) activate(s) the angiogenic switch, but undisputed that this is one very important step in clinical cancer development. This suggests that complete pharmacologic blockade of tumor angiogenesis will leave only residual microscopic, nonangiogenic lesions, which may be clinically harmless and manageable as a chronic condition *(6)*. Therefore, antiangiogenic therapies are increasingly being explored as an adjunct in cancer treatment, with the goal to (a) inhibit cancer growth and (b) reduce interstitial intratumoral pressure and thus improve drug delivery *(1)*.

The process of angiogenesis (and thus likely the effectiveness of antiangiogenic therapies) can be imaged with PET, for example, by using an ^{18}F-labeled RGD peptide, which binds specifically to $\alpha\nu\beta3$ integrin receptors expressed at the surface of proliferating endothelial cells. $\alpha\nu\beta3$ is a transmembrane glycoprotein receptor involved in tumor growth, local invasiveness, and metastasis. It recognizes the tripeptide sequence arginine–glycine–aspartic acid (RGD). Haubner et al. *(10)* developed an RGD peptide whose structure (conformation) is such that it binds specifically to the $\alpha\nu\beta3$ integrin receptor. Subsequently, the peptide was labeled with the positron emitter ^{18}F. Specific binding of ^{18}F RGD peptide was then confirmed in animal and human studies. Clinically, $\alpha\nu\beta3$ integrin expression has been imaged in the neovasculature of patients with melanoma, head and neck cancer, and breast cancer *(11–13)*. The intensity of ^{18}F RGD peptide accumulation correlates with the presence of activated endothelial cells and, quantitatively, with microvessel density *(10,12)*. It should be noted, however, that angiogenesis is not specific for cancer only. Instead it can also be observed in a number of benign, inflammatory processes such as villonodular synovitis *(12)*. Clinical studies are underway to see if imaging with this or similar compounds may predict and monitor the response to antiangiogenic therapies in a specific and quantitative manner. Although a hallmark of cancer, angiogenesis is indeed also a physiologic process in human development and reproduction (menstruation cycle), and also occurs as part of

many benign diseases (macular degeneration, psoriasis, endometriosis, and arthritis). Accordingly, ^{18}F RGD peptide (and likely any other radiotracer probing the presence of neovasculature) may also show accumulation in such benign conditions.

2. RECEPTOR EXPRESSION IN PROSTATE AND BREAST CANCER

Whereas cancer was traditionally thought of as a deregulated condition, a number of molecules, receptors, and pathways are now known whose presence and activation is in fact critical for cancer development and progression. This includes regulating proteins (such as HIF-1α), the Akt/PI-3-K or mTOR pathways, and a number of receptors (such as ErbB-2/Her2 and the androgen receptor, AR). The mere presence of a receptor, however, does not indicate that the receptor is indeed functional (active), essential to cancer growth and progression, or that pharmacological interference with receptor function will result in tumor cell kill. Moreover, analysis of receptor status is limited to the surgical or biopsy specimen. Efforts are, therefore, underway to develop noninvasive probes for imaging of receptor status and activity. Several PET tracers are now available for imaging of the androgen receptor (AR) *(14)*, estrogen receptor (ER) *(15,16)*, or ErbB-2 (Her2) receptor *(17)*.

Androgens, signaling through the AR, are the primary regulators of prostate cancer cell growth and proliferation. Historically, it was assumed that progressing prostate cancer eventually reaches a clinical state where further disease progression is androgen independent. Clinical and laboratory studies in the past 5–10 years have shown that this concept is not true. In fact, there is continued signaling through the AR in progressive prostate cancer despite castrate serum levels of testosterone, and this phenomenon continues into the late states of the disease *(18–20)*. Intratumoral androgens are also found even in late stages of the disease. AR acts through its "genomic function" (promoting protein synthesis) and also through a nongenomic function (acting as a signaling molecule and activating certain tumor pathways).

Because continued signaling through the AR occurs even in advanced prostate cancer and in fact promotes disease progression, pharmacologic studies are now underway to target the AR and cause its destruction or downregulation. Experimental studies using HSP-90 inhibitors *(21)*, RNA interference *(22)* or short hairpin RNA (shRNA) *(23)* have provided the proof of concept in this regard. (HSP-90 is a "chaperone" protein needed for the stabilization and function of the androgen receptor). Verification of the AR expression in patients with metastatic prostate cancer would require repeated biopsies from multiple sites. A noninvasive method for systemic and repeated study of AR expression and function would be preferred. It is therefore of increasing interest to develop a method to measure AR expression and activity. This should improve our understanding of prostate cancer biology and should be helpful in monitoring the response to drugs targeting this receptor. Potentially, such method could also identify prostate cancer patients more likely to respond to certain classes of drugs or small molecules targeting the androgen receptor. Among several compounds developed for this purpose *(24)*, the agent 16β-^{18}F-fluoro-5α-dihydrotestosteron (FDHT) is currently under clinical investigation for imaging and quantifying the androgen receptor expression in metastatic prostate cancer *(14,25)* (see Fig. 1). Initially, it was expected that an inverse relationship would exist between prostate cancer differentiation and AR expression, which should translate into a mismatch between FDG uptake (known to occur predominantly in aggressive metastatic lesions) and FDHT uptake (AR expression). Imaging studies, however, reveal various patterns of FDG and FDHT distribution in prostate cancer metastases, including the postulated inverse relationship, but also simultaneous high or low accumulation of both radiotracers in some lesions (Fig. 1). This is, at least in part, in keeping with the observation that the AR remains expressed and functional even in the late

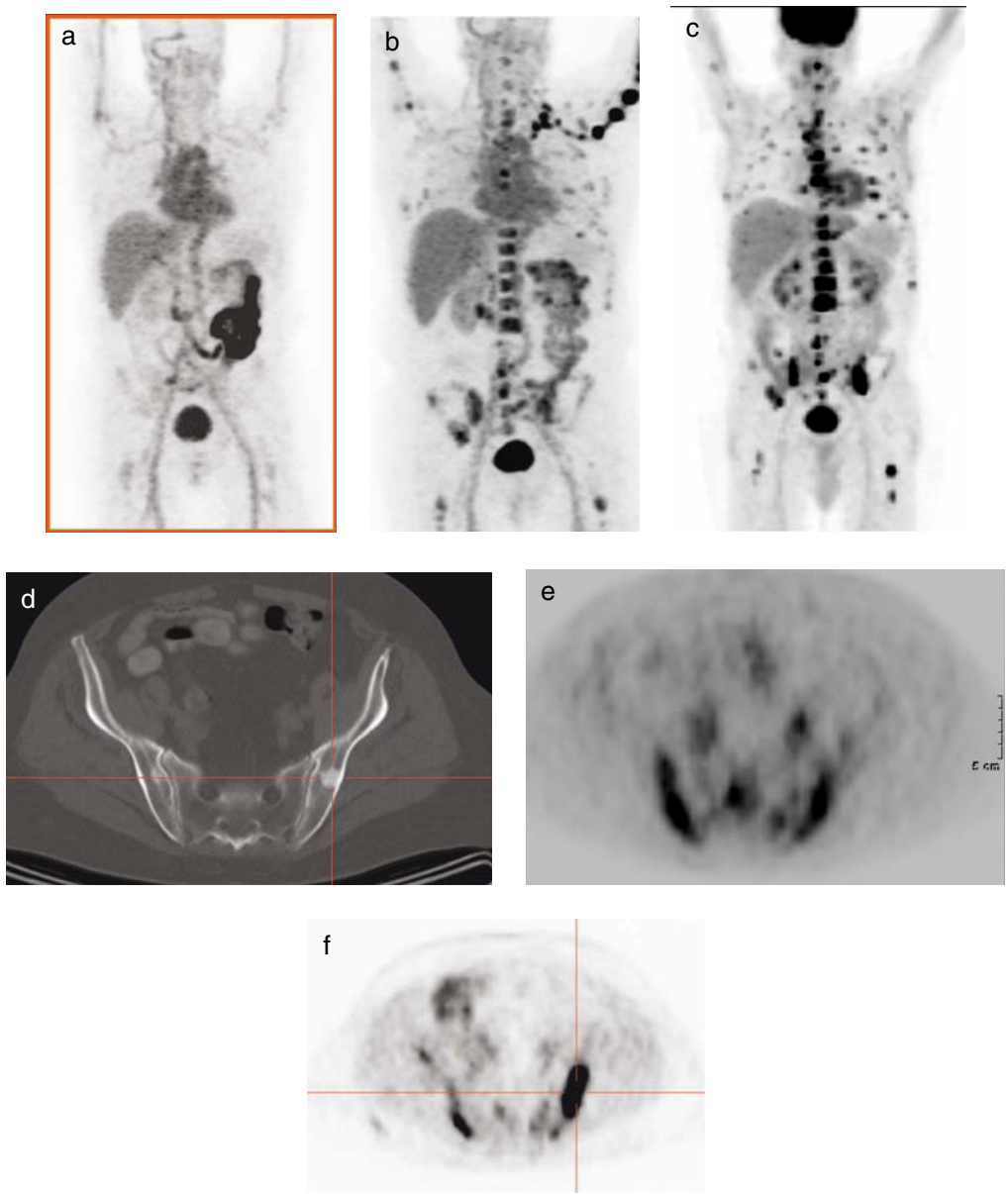

Fig. 1. Biodistribution of FDHT and FDG in metastatic prostate cancer. (**A**) Normal distribution of FDHT showing blood pool, hepatobiliary, and urinary tracts. (**B, C**) Maximum intensity projection images showing uptake of FDHT (**B**) and FDG (**C**) in osseous metastases from prostate cancer. (**D**) Transaxial CT image shows sclerotic metastasis in left iliac bone (*cross hairs*) and smaller lesion in the right iliac bone. (**E–F**) Transaxial FDHT (**E**) and FDG (**F**) images reveal abnormal radiotracer accumulation in pelvic bones that far exceeds the structural abnormality seen on CT, which indicates spread of metastasis throughout the bone marrow not detectable on CT.

clinical states of prostate cancer (note that this is in contrast to thyroid cancer, where increased FDG uptake signifies an advanced disease state in which the sodium–iodine symporter is no longer expressed and radioactive iodine therefore no longer taken up). Of note, recent in vitro studies show that AR expression indeed correlates with invasiveness in both *androgen-dependent* and

hormone-refractory cell lines which can be suppressed by blockade of the AR with shRNA *(26)*. Accordingly, one might expect that novel drugs specifically targeting the AR will be beneficial in men with advanced, hormone-refractory metastatic prostate cancer when standard antiandrogens are no longer effective, and that the response to such drugs could be monitored by FDHT PET. This is currently under investigation.

Most breast cancers are estrogen receptor (ER)-positive tumors and may thus potentially respond to antiestrogen therapy. Nevertheless, chemotherapy (rather than hormonal therapy with antiestrogens) is often used as the primary means of treatment in advanced breast cancer, in part because chemotherapy is expected to cause tumor shrinkage more reliably and more rapidly than hormonal agents. While ER expression on breast cancer is demonstrated by immunohistochemistry, the mere presence of the receptor predicts clinical benefit in only 55–60% of patients. Higher expression levels for both ER and progesterone receptors in cancer tissue improve the likelihood of response to tamoxifen and expand the time to treatment failure *(27)*. However, expression levels may vary between primary tumor and metastasis and may not predict the functional status of the receptor (active versus inactive); moreover, no blood test or structural imaging study can accurately predict for clinical benefit from hormonal therapy. The PET tracer [^{18}F]fluoroestradiol (FES) was therefore developed to assess ER expression and functional status noninvasively in women with advanced breast cancer.

In clinical studies, there is good agreement between the FES signal on PET images and estrogen receptor expression by immunohistochemistry *(16)*. FES PET might therefore be a tool to define the ER expression status on disseminated breast cancer lesions. One might also expect that the degree of FES uptake in breast cancer lesions may predict a response to hormonal therapy, but in preliminary clinical studies there was considerable overlap in the FES PET signal between patients showing clinical response to Tamoxifen and nonresponders. Interestingly, a phenomenon termed "metabolic flare" predicted the treatment response much better than a positive signal on FES PET *(15)*. "Metabolic flare" is defined as the transient increase in FDG uptake in breast cancer lesions as compared with baseline during the early phase of treatment with antiestrogens. Therefore, PET imaging with both FDG and FES is now part of ongoing clinical trials to determine how early and accurately a response to hormonal therapy in breast cancer can be predicted and monitored.

3. GENE EXPRESSION IMAGING

Gene therapy of malignant tumors is based upon the transduction of cancer cells with a therapeutic gene, using a vector for gene delivery into the cancer cell. The expression of such therapeutic genes may render the cell sensitive to prodrugs (suicide gene therapy), alter the expression of cell-cycle-regulating proteins, induce apoptosis or stimulate an immune response. Only a small number of gene therapy trials have been conducted in cancer thus far, and their results have been largely disappointing *(28,29)*. Suboptimal gene delivery techniques, short duration of gene expression, insufficient distribution of vector particles throughout the entire tumor, changes in the tumor microenvironment and activation of alternate tumor pathways may be some of the reasons for this. Cancer trials with stem cells or progenitor cells have also disappointed showing at best transient responses *(30)* (although promising results have been reported for other diseases *[31,32]*). Importantly, most of these trials were conducted "blind," using assessment of function (in heart failure trials) or lesion size (in cancer trials) as indirect "surrogate" measures for gene expression. These indirect approaches are clearly suboptimal, and thus it would be desirable to assess gene expression levels and the distribution of transfected cells in the human body in real time. Indeed imaging of gene expression is feasible and has been

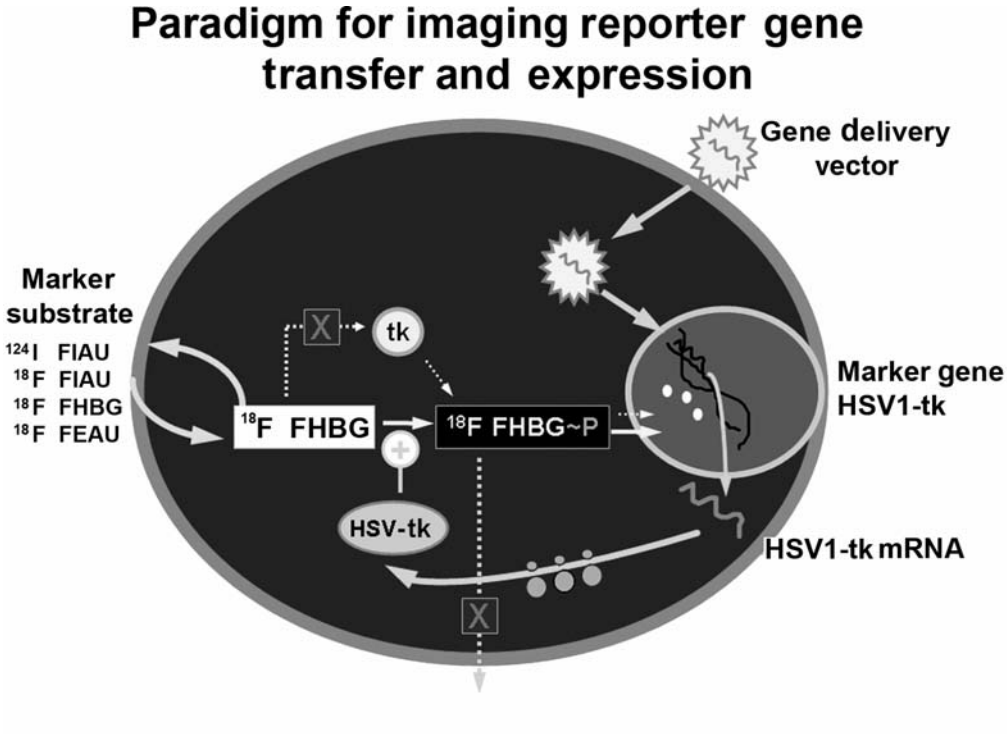

Fig. 2. Schema showing the principle of gene expression imaging; see text for explanation (modified from Gelovani, Blasberg et al., Cancer Res 1995; 55:6126).

studied as part of preclinical studies for the past 10 years *(33)*. The imaging is done "indirect," using the combination of a "reporter probe" and "reporter gene." One simple example is the use of HSV-tk, whose expression causes synthesis of the protein thymidine kinase, which in turn phosphorylates PET tracers (such as FIAU, FEAU, or FHBG) causing their specific retention only in cells that express the HSV-tk gene *(34,35)* (see Fig. 2). It should be pointed out that other modalities, in addition to PET, are also very well suited to image gene expression and cell transfer *(36)*. Potential clinical applications include

- the assessment of the transduction efficacy (location, extent, and duration) and quantitative monitoring of gene expression in gene therapy protocols as shown exemplary in a small clinical study *(37)* and
- the monitoring of cell trafficking, for example, T cells used for adoptive immunotherapy *(38,39)*.

Experimental studies have proven that it is feasible to visualize transfected embryonic stem cells in ischemic hearts using bioluminescence imaging *(40)* or transfected mesenchymal stem cells in animal models of nephropathy *(41)* and glioma *(42)* by using immunofluorescence imaging as well as MRI (SPIO). In future clinical trials, the tracking of stem cells or T cells (biodistribution and gene expression levels) will be an important tool to understand reasons for treatment failure and improve the response rates in such trials. This will require coexpression of the reporter gene with the therapeutic gene, so that the imaging signal not only reflects cell distribution but also the function of the therapeutic gene. The feasibility of this approach was recently shown in an experimental glioma model *(43)*. A major shortcoming of many experimental studies is the fact

that therapeutic genes (or cells carrying such genes) are delivered locally into tumor-bearing tissue. Because cancer is a systemic disease, this will not be successful in clinical practice. Instead, in future cancer trials, one would like to image cells that are administered systemically (i.e., intravenously, rather than selective into tumor-feeding vessels or into tumor tissue directly).

In summary, many functions of the cancer cell can be imaged noninvasively. PET is a tool to study the receptor expression on the cancer cell as well as mechanisms of invasion and angiogenesis. This may be useful for diagnostic purposes as well as in monitoring the response to therapies specifically attacking these traits. Gene expression can be imaged, and it is expected that this will be an important element in future gene therapy trials.

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11 MRI and Ultrasound Imaging of Lymph Nodes

Marika Decyk, BS, Yuko Kono, MD, PhD, and Robert Mattrey, MD

CONTENTS

ABSTRACT

Developments in MR and ultrasound imaging technology combined with novel contrast media allows the detection of sentinel lymph node(s) and potentially the distinction of benign from malignant nodes. These advances could reduce dissection during sentinel lymph node resection to a minimally invasive procedure and could allow the assessment of whether sentinel nodes harbor metastatic deposits. Targeted contrast media is on the horizon and promises to identify patients who need sentinel lymph node resection, detect the sentinel node with greater specificity, and detect the presence of micrometastases.

In this chapter, we will describe the mechanism of lymph node enhancement with contrast media given intravenously or subcutaneously, as well as present the current status of MR and ultrasound imaging of lymph nodes.

Key Words: lymph node; sentinel lymph node; tumor; CT; ultrasound; MRI

1. INTRODUCTION

Lymph node status remains the most important prognostic indicator of cancer outcome because it defines cancer aggressiveness and extent of spread. Traditional lymphography assessed nodal involvement in cancer via direct cannulation of a foot lymph duct and slow injection of a lipid-based iodinated contrast agent that remains within the lymph vessels to fill the sinusoidal space of the nodes. Metastatic deposits could be seen on plain radiographs as filling defects

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within the node. This technique has been abandoned because it was time consuming and limited to the lymph chain that drains the feet. Imaging lymph nodes to define their benign or malignant status remains an important goal and challenge. For instance, in breast cancer patients, axillary lymph node dissection and histological assessment of the nodes has been the gold standard for determining patient prognosis and optimal therapy. While axillary node dissection is the major cause of postoperative complications in breast cancer surgery, axillary nodes are positive in only 30–60% of patients (*1*). Should axillary dissection be limited to those who need it, 40–70% of patients could be spared complications.

A new technique, sentinel lymph node resection, was introduced to decrease the rate of post-operative complications following traditional axillary node dissection (*2*). It requires the identification, removal, and immunohistochemical analysis of the first lymph node to which the metastatic cells drain. Although still invasive, it has decreased morbidity and has become a common method for assessing lymph node involvement. This new approach has added a new challenge to lymph node imaging. In addition to assessing whether nodes are benign or malignant, imaging must now also recognize the sentinel node from all the nodes draining the tumor.

In this chapter, we will describe the principles underlying the various imaging approaches as well as their advantages and limitations. Since all current lymph node-imaging techniques are limited, we will focus on techniques nearing or in clinical development, as recent advances in imaging, and contrast media have made it possible to specifically image lymph nodes. We will focus on MR, CT, MRI and Ultrasound Imaging of Lymph Nodes. Although the imaging principles for nuclear techniques are similar, they will be covered elsewhere in this book.

2. MECHANISM OF LYMPH NODE ENHANCEMENT

Lymph nodes are supplied by both blood and lymph flow and consist of two major compartments: the cellular compartment containing phagocytes and the sinusoidal compartment filled with lymph (Fig. 1). The mechanism of lymph node enhancement varies with the size of the molecule/particle injected and the mode of administration. In clinical practice, the currently available small molecular weight contrast media for CT and MRI enhance lymph nodes through both the vascular supply to the node and the lymphatic channels. Vascular enhancement dominates when nodes are enlarged because of inflammation or when replaced by metastatic deposits, while lymphatic enhancement dominates when nodes are normal or harbor small metastases. Except for the timing of enhancement with vascular occurring earlier than with lymphatic, which can be assessed with dynamic scanning, the pattern of enhancement is indistinguishable. Lymphatic enhancement with current contrast agents given intravenously occurs rapidly because of the rapid leak of the small water-soluble molecules into the interstitium. This is followed by rapid entry of the molecules into the lymph vessels that carry them to the sentinel as well as all other nodes in the drainage field and ultimately to the thoracic duct. Should these agents be injected directly into the interstitium, the draining nodes enhance intensely (*3*).

Unlike the small molecular weight water-soluble agents, particulate contrast media, especially when larger than 10–20 nm in diameter, leak across normal capillaries and enter into the lymph vessels very slowly—hours to days depending on particle size *(4,5)*. On average, smaller particles (10–20 nm) are more likely to enter than larger particles. As particles approach 1 μm, their entry into the lymph vessel is very poor because they must be carried away by phagocytes or reduced in size by local processes. In fact, over 95% of particles larger than 400 nm stay at the injection site, whereas 74% of particles 10 times smaller (40 nm) are absorbed (*6*). Particles that either leak across capillaries or are injected directly into the interstitium in turn accumulate in phagocytes in

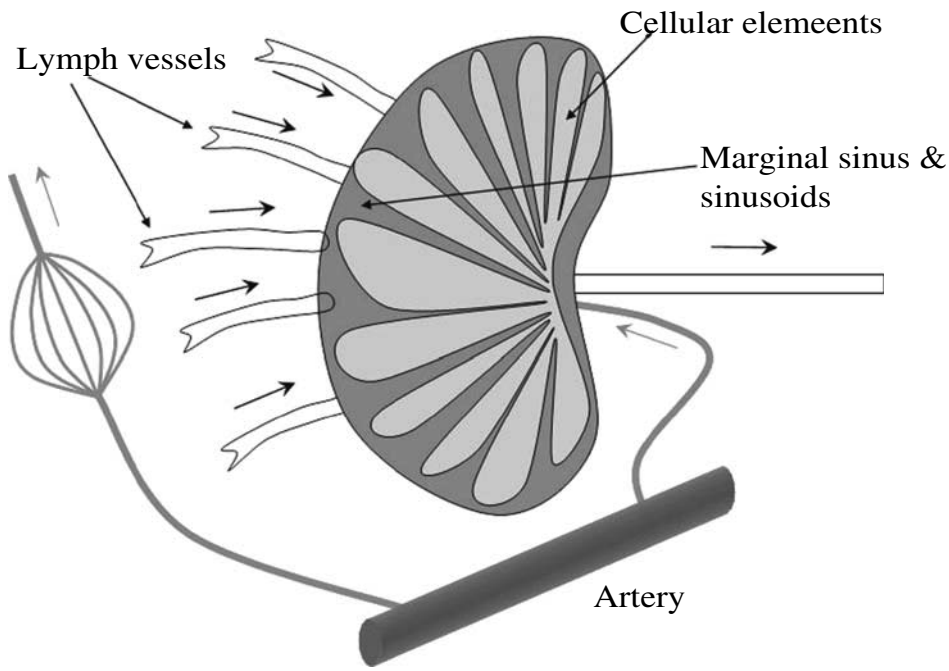

Fig. 1. Cartoon depicting lymph node structure and the relation with blood vessels and lymph vessels. The lymph nodes' connection to both vascular and lymphatic vessels allows either intravenous or subcutaneous injections of molecules/particles to reach lymph nodes. Contrast agents given systemically leak across capillaries, enter lymph vessels, and enhance all nodes. Contrast media given interstitially in a drainage bed enter the lymph to enhance the sentinel and/or all local nodes.

the draining nodes (*5*) that either carry them directly from the injection site or trap them should they enter the lymph vessel. Transport of larger particles (>200 nm) away from an injection site is dominated by cellular transport (*7*). These particles are typically injected directly into the interstitium since their probability to leak across normal capillaries is very low.

3. SENTINEL NODE DETECTION

We will focus here on CT, MR, and ultrasound techniques to detect lymph nodes, since nuclear techniques— and, particularly, the macrophage-targeted agent Lymphoseek that is entering Phase III clinical trials— are covered elsewhere in this book. Since intravenously injected particulate contrast media reaches lymph nodes predominantly through the lymph after leaking across capillaries, they enhance all nodes throughout the body. The intravenous route of administration is therefore more suitable for lymph node mapping and the distinction of benign from malignant nodes. To detect the sentinel node in a tumor's drainage field, contrast media must be injected directly near the tumor or in its drainage field. When water-soluble small molecular weight contrast media such as iodinated CT agents and Gd-based MR agents are injected subcutaneously, their pharmacokinetics mimic those of the blue dye used in sentinel node resection. Immediately following injection, depending on the concentration and dose used, the agent enters the lymph and enhances ducts and nodes; this provides a lymph node map of the drainage field (*3*). The rapid enhancement of the secondary lymph nodes down the chain makes it difficult to recognize the true sentinel node.

Gd-based macromolecular agents injected subcutaneously slow the transit rate through the lymph as compared with the small molecular weight agents, making their use for sentinel node detection by MRI more viable (*8–10*); however, none of these agents are currently available for clinical testing. One small molecular weight water-soluble agent with high protein binding is in late-phase clinical development as an intravenous blood pool agent, and could potentially function as a macromolecular agent for lymph node imaging (*11*). Radiopaque particles for CT *(5,7)*. and iron oxide nanoparticles (IONP) for MRI (*12*) can be injected directly into the interstitium to image nodes in the drainage field. Once injected, particles are detected in the phagocytes of the draining nodes, but the lymph duct is not visualized, likely because few particles are present in the duct due to the slow transit rate. Although MRI can detect much smaller concentrations of IONP than CT can detect radiopaque particles, IONP decreases rather than increases MR signal, making the lymph duct difficult to recognize. In the case of IONP that is black in color, the clearance rate from the injection has to be defined to ensure that IONP can be cleared.

Standard microbubbles, 1–3 μm in diameter, are unique particles that can enter the lymph when injected into the interstitial space because of their ability to deform *(13,14)*. Because microbubbles interact with ultrasound differently than tissues, contrast-specific imaging instrumentation has afforded ultrasound extreme sensitivity to microbubbles, allowing the recognition of a single moving microbubble (*15*). When a microbubble agent that is FDA-approved for intravenous use during echocardiography was injected subcutaneously and the injection site massaged, a sufficient number of microbubbles entered the lymph immediately to allow the visualization and tracking of the lymph duct from the injection site to the first draining node (sentinel node) in real-time (Fig. 2) (*13*). The sinusoidal space of the draining node filled completely and enhanced the entire node *(13,16,17)*. It is not yet clear whether targeted or specially designed microbubbles can be trapped in the sentinel node to limit the visualization of downstream nodes.

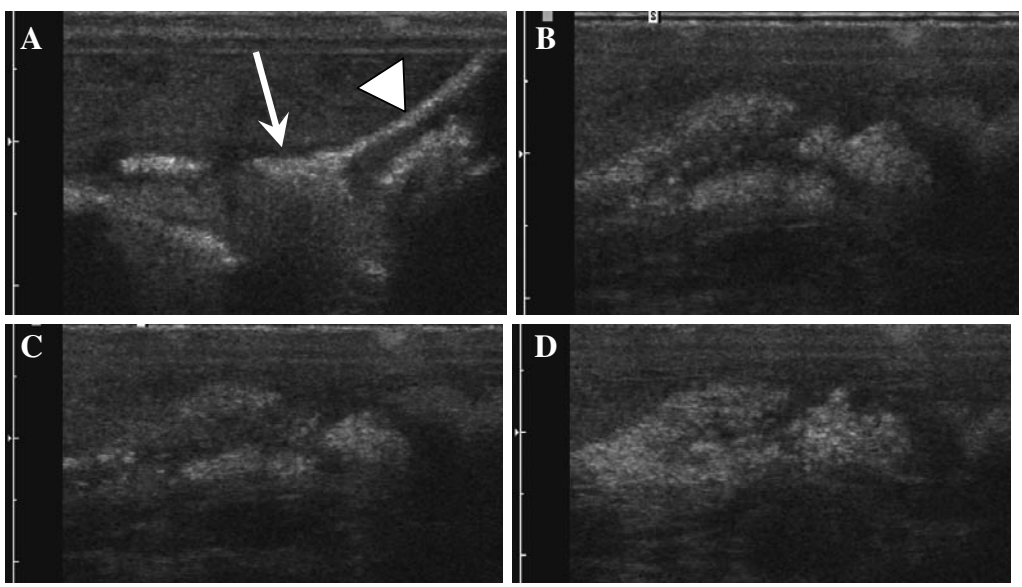

Fig. 2A–D. Images of the iliac and retroperitoneal lymph nodes obtained with intermittent imaging (5-sec interframe delay) are shown (**A**) early, and (**B–D**) late during filling. (**A**) Shortly after massage, the lymph duct (*arrowhead*) is seen leading to the enhanced node (*straight arrow*). (**B–D**) Images obtained after a 20-sec delay show complete filling of nodes.

Another unique feature of microbubbles is that they are destroyed when imaged with clinical ultrasound instruments *(18,19)*. Their elimination from the imaged field provides the opportunity to study the refill rate and pattern within the region of interest. In the case of lymph node imaging, remassaging the injection site allows the tracking of the same lymph duct to the same node to confirm that the node identified is the first node draining the injection site, or tracking a different lymph duct to another node to document the presence of a second sentinel node. While some formulations allowed the refilling of ducts and their draining node up to 12 times following a single 0.5-ml subcutaneous injection *(14)*, newer formulations have allowed up to 20 refills from a single 0.5-ml injection [data not yet published]. Ultrasound's ability to visualize the lymph duct and track it noninvasively to the draining nodes is a powerful technique that will undoubtedly impact sentinel node detection and resection. While microbubbles are FDA approved, no clinical trials have been initiated for this indication.

With all sentinel node detection techniques, it should be noted that when metastases totally replace a lymph node, lymph can be redirected away from the sentinel node *(20)*; however, such node(s) will likely be enlarged and should be easily detected by the sonographer.

4. RECOGNITION OF BENIGN FROM MALIGNANT NODES

Benign nodes have the expected fatty hilum with a thin cortical rim and marginal sinus. However, should such nodes harbor micrometastases in or near the marginal sinus, they will likely be missed. When the fatty hilum is lost and the node is enlarged, it is difficult to distinguish benign from malignant nodes on CT and MR unless intranodal necrosis is detectable following intravenous contrast administration. Standard noncontrast-enhanced ultrasound can assess enlarged nodes and, based on their size, shape, echogenicity, and morphology, can recognize metastatic from benign lymph nodes. When used in combination with fine needle aspiration biopsy, ultrasound's sensitivity ranges from 57 to 80% *(21)*. When microbubbles are given intravenously, they are limited to the vascular space because of their 1- to 3-μm diameter; however, because ultrasound can visualize the arrival of contrast media to the node of interest in real time, an angiographic assessment of the node is possible aiding in the distinction of benign from malignant nodes. This hypothesis has not yet been tested clinically. When microbubbles are given interstitially they fill the entire node. Should metastatic deposits exist, they will appear as filling defects, allowing their recognition (Fig. 3) *(13)*.

Ultrasmall superparamagnetic iron oxide particles (USPIO) are approximately 50 nm in diameter and are coated with low molecular weight dextran. When given intravenously, they slowly leak across normal capillaries and enter the lymph vessel to be carried into nodes where they are readily phagocytosed by lymph node macrophages. Since IONP dramatically shortens T2 relaxation to reduce the MR signal of nodes on T2-weighted images, it promotes the detection of metastatic deposits that are bright on T2-weighted images because the latter displace the normal internal architecture that was made dark by IONP (Fig. 4) *(22)*. The added image contrast between normal nodes and metastatic deposits increased sensitivity from 35% to 90% and found all patients with metastatic nodes *(22)*. Since USPIO is given intravenously, it darkens all nodes and allows for lymph node mapping *(1)*, but cannot allow the distinction of the sentinel node(s) from all other regional nodes.

The data reported for prostate cancer patients were also reproduced in breast cancer patients as reported by Memarsadeghi et al. *(1)*, who found that USPIO allowed for an accuracy of 98% on a node-by-node basis and 100% on a patient-by-patient basis. The study also found that when qualitative assessment of an image was questionable, quantitative measurement of the decrease in signal intensity was sufficient to differentiate normal from metastatic lymph nodes. Given

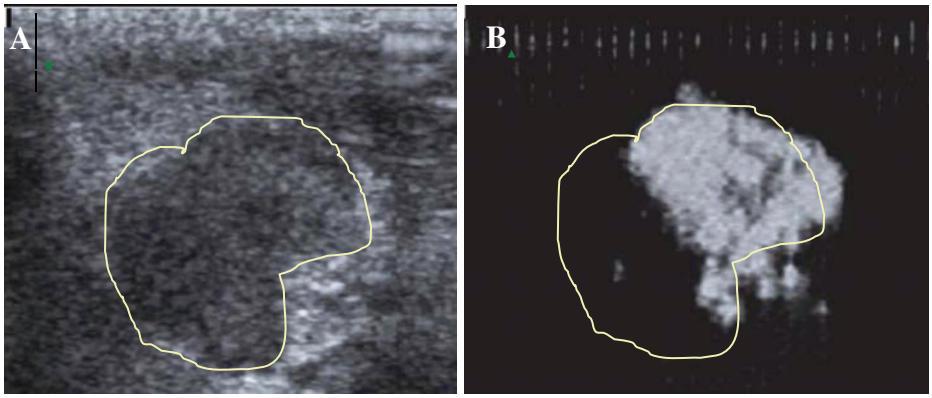

Fig. 3 (**A** & **B**) Ultrasound images acquired with a standard ultrasound technique (**A**) or with harmonic imaging (**B**) of the popliteal node of a rabbit with Vx2 tumor in the calf show the filling of only the normal portion of the node (**B**). The entire node is seen with standard ultrasound [overlay in (**A**)]. Note that with harmonic imaging that severely suppresses tissue signal and shows only microbubbles (**B**), only the normal portion of the node fills with contrast. (Adapted from Fig. 3 in (*13*) with permission.)

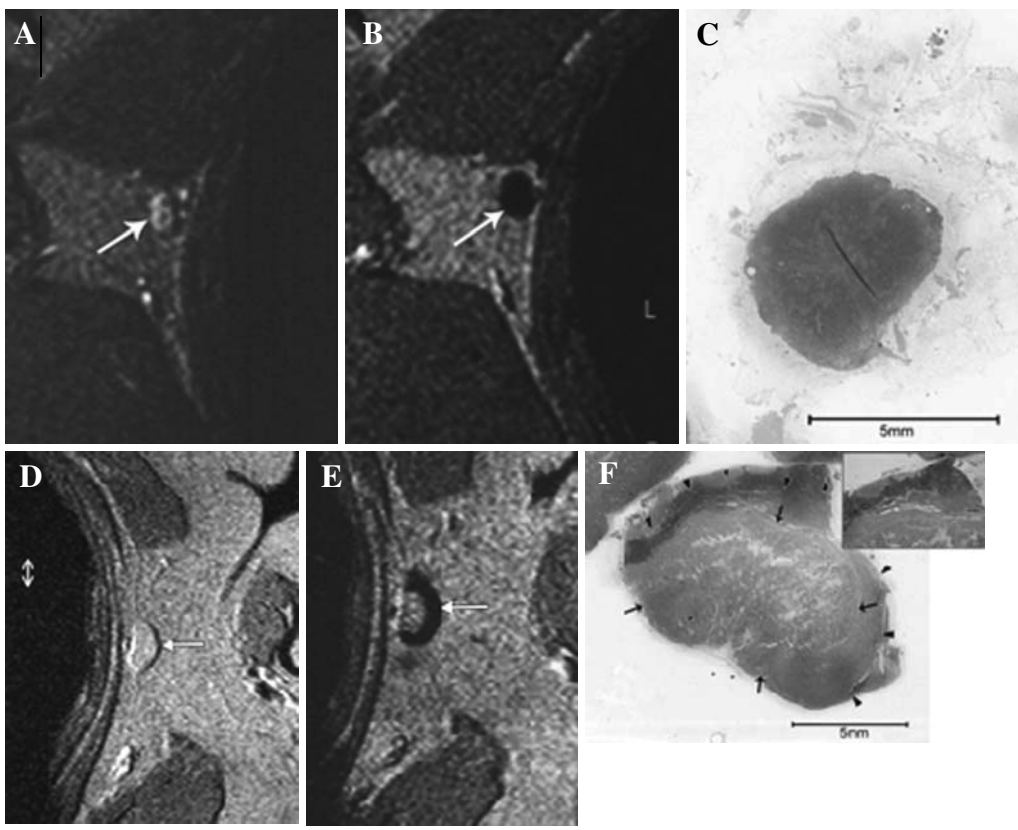

Fig. 4 (**A–C**) Case 1: (**A**) Nonenhanced and (**B**) 24–36 hours after intravenous injection of USPIO, transverse T2*-weighted FFE MR images show uniform SI decrease after USPIO administration. (**C**) Photomicrograph of histopathologic specimen shows node with normal appearance. (Adapted from Fig. 2 in *(1)* with permission.) (**D–F**) Case 2: (**D**) Nonenhanced and (**E**) USPIO-enhanced T2*-weighted FFE transverse MR image (683/14) shows the lymph node with partial SI decrease, which is indicative of metastatic involvement. (**F**) Photomicrograph of histopathologic specimen of same lymph node confirms presence of metastatic tissue (*arrows*). Peripheral zone is nonmetastatic tissue (*arrowheads*). (Adapted from Fig. 4 in *(1)* with permission.)

the spatial resolution at which images can be acquired in the axilla using surface coils, USPIO-enhanced MR imaging could potentially resolve a 1-mm deposit. USPIO is not yet approved for clinical use. The larger particle size iron-oxide preparation Feridex that is approved for liver imaging is too large (250 nm) to be useful for lymph node imaging.

Targeting contrast media to lymph endothelial cells, phagocytes, or malignant cells within lymph nodes is possible. Such an approach for MR and ultrasound imaging has not yet been reported.

While noninvasive imaging can potentially screen for lymph node involvement, the false-positive rate is not yet known. Until such time when imaging can provide very high negative and/or positive predictive values, histological analysis requiring resection or biopsy will remain the standard clinical test.

5. CONCLUSION

Noninvasive imaging to detect and potentially assess sentinel lymph nodes is possible. Early data using either intravenously or interstitially administered novel contrast media are promising. Nanoparticles administered systemically accumulate within phagocytes in all lymph nodes, dramatically increasing the ability to distinguish between normal and malignant nodes. Novel contrast media and microbubbles injected near the tumor or in its drainage field allow the detection of the draining lymph ducts and sentinel node, which in turn may improve sentinel node detection and potentially minimize the dissection required to localize and resect sentinel lymph nodes.

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12 Molecular Imaging of the Sentinel Lymph Node via Lymphoseek

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Contents

ABSTRACT

Sentinel lymph node biopsy is performed with increasing frequency in patients undergoing surgery for early-stage cancer to avoid unnecessary lymphadenectomy in patients whose sentinel node is negative for metastatic disease. Mapping of the sentinel node via nuclear imaging involves the injection of a radiotracer into the vicinity of the tumor and the subsequent localization of the sentinel node by lymphoscintigraphy and gamma probe detection. Lymphoseek is a new radiotracer specifically designed for sentinel node mapping. It exhibits properties that are advantageous over other radiotracers such as filtered technetium-99m sulfur colloid. Preclinical studies demonstrate improved pharmacokinetic qualities and reduced radiation exposure, when using Lymphoseek compared with radiocolloids. Phase I and Phase II clinical trials conducted in breast cancer and melanoma patients confirmed Lymphoseek's safety in patients and improved identification of the sentinel lymph node. Preclinical studies also demonstrate potential applications of Lymphoseek in patients with gastric, colon, or prostate cancer. Additionally, Lymphoseek can be applied in minimally invasive procedures, which is currently becoming an essential technique in surgery.

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Key Words: sentinel lymph node biopsy; molecular imaging; radiopharmaceutical; Lymphoseek

1. INTRODUCTION

The technique of sentinel lymph node biopsy is common practice in the treatment of many solid tumors, including breast cancer and melanoma *(1–4)*. The challenge is no longer whether sentinel node detection is feasible and reliable but about how the procedure can be improved and its impact on clinical management *(5)*. The current radiopharmaceuticals available for sentinel node imaging were never specifically designed for this use but were brought to the patient when the technique was first described. The current agents have no target receptors on the sentinel node and offer no binding capabilities *(6)*. As such, what is in use today simply "drains" through a particular lymphatic system with uptake to many unnecessary downstream lymph nodes. Molecular imaging of the sentinel node offers detection of the appropriate node(s) via binding mechanisms specific to the agent and the node *(6)*. Thus, the sentinel nodes that are detected are truly the "first most important nodes" and not just the stream of nodes that the radiopharmaceutical has drained through. Lymphoseek is a new molecular imaging agent for sentinel node detection.

2. BACKGROUND

Identifying the sentinel lymph node with high precision is crucial for monitoring cancer progression *(6)*. If the sentinel node is negative for metastatic disease, the patient is very unlikely to have distal nodal or distant metastatic disease. It has been shown that performing a sentinel lymph node biopsy alone has no adverse effect on the survival of patients with breast cancer *(3)*. Localization of the sentinel node can be successfully achieved by mapping the lymphatic system with either blue dye or radioactivity or both combined *(7,8)*. A study evaluating the success rate of identifying sentinel nodes in melanoma patients has shown that using blue dye alone, using lymphoscintigraphy alone, or using both methods combined, sentinel lymph nodes were detected in 70, 84, and 96% of cases, respectively *(9)*.

Both blue dye and particulate radiotracers are not specifically designed for application in the lymphatic system. This poses several challenges. Blue dye diffuses passively into the lymphatic system and can clear quickly from the lymph nodes. Particulate radiotracers enter the lymphatic system by passive diffusion, but clear slowly from the injection site due to their large diameter. Additionally, particulate radiotracers can result in uptake in the distal lymph nodes, not only in the sentinel node *(8,10,11)*.

The commonly used blue dye in the USA is Lymphazurin (isosulfan blue), and Patent Blue V is available in Europe and Australia. Blue dye requires intraoperative administration for optimal visualization, which occurs within minutes of a subdermal or a peritumoral injection. Rarely, Lymphazurin can cause severe anaphylactic reactions *(12)*. One study, for example, documented anaphylactic reactions in 1.1% of 639 consecutively performed sentinel lymph node biopsies for breast cancer *(13)*.

In contrast to direct visualization of the sentinel node when using blue dye, nuclear imaging monitors the pathway of the injected molecules via lymphoscintigraphy or intraoperative gamma probe detection *(14,15)*. Three different types of agents are commonly used for this purpose *(8)*. The radiotracer used in Europe is technetium-99m-HSA nanocolloid (diameter of 4–100 nm), technetium-99m-antimony trisulfide (diameter of 3–30 nm) is available in Australia and Canada, and the standard radiopharmaceutical in the USA is filtered technetium-99m sulfur colloid (diameter of 50–200 nm) *(8)*. technetium-99m sulfur colloid was originally designed for liver

imaging and not for lymphatic imaging *(16,17)*, and its large diameter causes its entrance into the lymphatic system to be relatively slow *(18)*. Therefore, to achieve an acceptable identification of the sentinel nodes, early administration, at least several hours before the surgery, is required *(8)*. Another disadvantage of technetium-99m sulfur colloid as well as the other radiolabeled particulates is the difficulty to exactly reproduce the same particle concentration, because the particles are created in the vial during the radiolabeling process and may additionally change after filtering. Due to the shorttime period between the preparation of technetium-99m sulfur colloid and its application in the patient, it is not possible to measure its particle concentration as there is no known rapid method for counting small particles within the technetium-99m sulfur colloid injectate.

3. OPTIMAL TRACER

Given the inherent challenges in blue dye and radiolabeled particulates, a new set of attributes was proposed *(19)*. The optimal sentinel lymph node tracer is one that rapidly clears from the injection site and does not interfere with the detection of the adjacent sentinel node. This property will also improve radiation safety by decreasing radiation exposure to the patient and the health care personnel. The radiation risk of the agent in general should be low, and it should not yield any local or systemic toxicity in the patient. The tracer should also have a rapid uptake, so as to not extend the time of surgery. Furthermore, it is highly desirable for the agent to exhibit a prolonged retention in the sentinel node which allows for more flexibility in scheduling of the imaging. Most importantly, it should accumulate only in the sentinel node and not in distal lymph nodes, since this assures accurate identification of the sentinel node and permits a more focused histopathologic analysis.

4. LYMPHOSEEK

Lymphoseek (Fig. 1) is a new radiopharmaceutical that was specifically designed for sentinel node mapping by lymphoscintigraphy and gamma probe detection. It is a macromolecule comprising a dextran backbone to which multiple units of mannose and DTPA are covalently attached *(18)*. Lymphoseek has a diameter of 7 nm and is, therefore, significantly smaller than

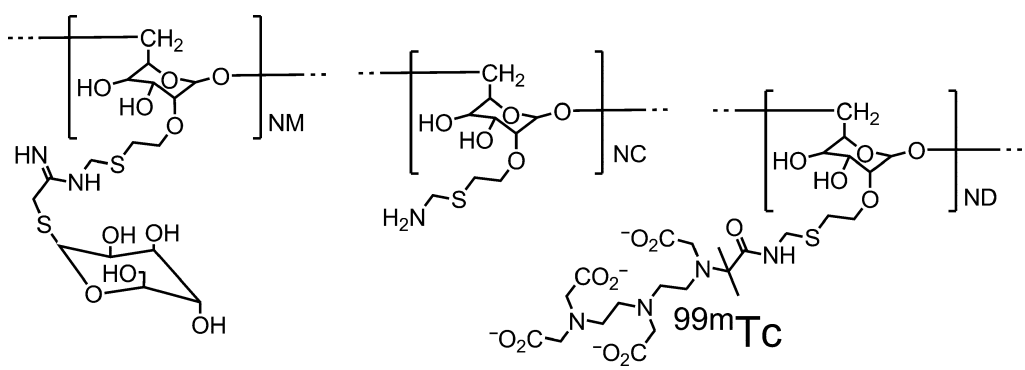

Fig. 1. Molecular structure of Lymphoseek. The macromolecule comprises a dextran backbone to which multiple units of mannose and DTPA are synthetically attached, enabling a specific uptake in the lymphatic tissue and a high accumulation in the sentinel lymph node.

any of the radiocolloids *(18)*. The small diameter permits it to rapidly enter the blood capillaries and the lymphatic channels, which results in a rapid clearance from the administration site. The DTPA units enable radiolabeling with technetium-99m while the mannose units function as a substrate that binds Lymphoseek to a receptor located on the surface of macrophages *(19,20)*. This makes Lymphoseek an ideal agent to be used in the lymphatic tissue since macrophages accumulate in the lymph nodes, especially in the sentinel node, in order to act as a barrier to migrating cancer cells.

5. PRECLINICAL STUDIES

Preclinical studies have demonstrated Lymphoseek's advantageous characteristics of an optimal radiotracer. The purpose of these studies was to evaluate Lymphoseek for its safety and pharmacokinetics, and consequently to assess Lymphoseek for applicability in various tumors and with different surgical techniques. Three pharmacokinetic properties of Lymphoseek were characterized: the clearance of the injection site, the accumulation in the sentinel node, and the retention in the sentinel node.

The injection site clearance was evaluated by conducting biodistribution studies in rabbits to prove that Lymphoseek exhibits a faster injection site clearance than filtered technetium-99m sulfur colloid. After Lymphoseek was injected subdermally into the foot pad of the rabbits, it showed a clearance half-life of 2.21 ± 0.27 hours at the site of administration *(21)*. The injection site clearance of Lymphoseek was significantly faster ($p < 0.05$) than that of filtered technetium-99m sulfur colloid, confirmed by a percent of injected dose (%ID) of 52.6 ± 10.5 at 1 hour and 45.7 ± 8.5 at 3 hours after administration of Lymphoseek versus 70.4 ± 11.0 at 1 hour and 55.5 ± 7.8 at 3 hours after administration of filtered technetium-99m sulfur colloid *(18)*.

To prove that Lymphoseek also clears rapidly from the injection site when administered endoscopically into the gastrointestinal tract, porcine studies were conducted comparing the injection site clearance of Lymphoseek versus filtered technetium-99m sulfur colloid. Lymphoseek exhibited a statistically faster ($p = 0.03$) clearance half-life compared with filtered technetium-99m sulfur colloid when endoscopically administered into the stomach and colon *(22)*. After submucosal injection into the stomach and colon, Lymphoseek revealed a clearance half-life of 3.83 ± 1.18 and 2.56 ± 1.04 hours, respectively, while filtered technetium-99m sulfur colloid exhibited a clearance half-life of 14.52 ± 4.08 hours in the stomach and 14.98 ± 3.41 hours in the colon *(22)*.

Lymphoseek's rapid accumulation in the sentinel node was demonstrated in porcine studies in which Lymphoseek or filtered technetium-99m sulfur colloid were endoscopically administered into the stomach and colon. Although the sentinel node uptake of Lymphoseek after 3 hours was not significantly different from the uptake of filtered technetium-99m sulfur colloid ($p = 0.88$ for gastric lymph nodes and $p = 0.67$ for colonic lymph nodes), the uptake of Lymphoseek into the sentinel lymph node was very high within 10 minutes of gastric and colonic submucosal injection (0.13–4.5% ID for gastric lymph nodes and 0.54–2.4% ID for colonic lymph nodes) *(22,23)*, A rapid accumulation in the sentinel node was also shown in porcine prostatectomy studies in which sentinel lymph nodes could be detected by a gamma probe within 20 minutes after peritumoral injection *(24)*.

Lymphoseek was retained longer in the sentinel node than filtered technetium-99m sulfur colloid in preclinical studies with rabbits. In these studies, the sentinel node was represented by the popliteal lymph node of the rabbit and the distal lymph nodes were represented by the inguinal lymph nodes. To evaluate the retention in the sentinel node, the sentinel lymph

node extraction was determined by measuring the radioactivity of the sentinel node relative to the distal lymph nodes. Sentinel lymph node extraction at both 1 hour and 3 hours postinjection was significantly higher ($p < 0.05$) for Lymphoseek ($90.1 \pm 10.7\%$ and $97.7 \pm 2.0\%$, respectively) than for filtered technetium-99m sulfur colloid ($78.8 \pm 6.5\%$ and $67.4 \pm 26.8\%$, respectively) *(18)*. This showed that Lymphoseek was retained longer in the sentinel node than filtered technetium-99m sulfur colloid, demonstrated by a higher sentinel node extraction, particularly after 3 hours. A longer retention of Lymphoseek in the sentinel node was also demonstrated in porcine prostatectomy studies in which Lymphoseek was sustained in the sentinel node for up to 3 hours without being washed out into distal lymph nodes *(18,22,24)*.

These pharmacokinetic properties of Lymphoseek result in several clinical advantages in the sentinel node detection. Lymphoseek's faster injection site clearance permits an improved visualization of the sentinel node, due to a reduced amount of scatter when localizing the sentinel node in the diagnostic image. Furthermore, this property decreases the radiation exposure to the patient and health care personnel *(18)*. The rapid accumulation in the sentinel node provides a time frame for administration of Lymphoseek between 5 minutes and 3 hours, making it a versatile radiotracer to use in minimally invasive surgery as it allows for intraoperative injection without extending the duration of surgery. This creates more flexibility for the surgeon regarding the time of injection and the time to correctly locate the hot nodes *(22)*. The ability of Lymphoseek to bind only to the sentinel node without being washed out into distal lymph nodes facilitates more precise imaging of the sentinel node, and may decrease the number of lymph nodes to be excised.

In summary, preclinical studies have shown that Lymphoseek is applicable for both open and laparoscopic surgeries, the latter made possible by using a specifically designed gamma probe that is attached to laparoscopic devices, enabling the surgeon to detect only the sentinel lymph node without interfering with the injection site radioactivity *(25)*.

6. BIODISTRIBUTION AND TOXICITY

Preclinical safety studies performed on rabbits demonstrated that Lymphoseek possesses an acceptable biodistribution *(18,21)*. Blood samples and dissected organs were assayed for technetium-99m activity using a gamma counter. The percent of injected dose was then calculated, followed by the calculation of the biological half-life of the injection site clearance which revealed a biological half-life of 2.21 ± 0.27 hours *(21)*. Compared with filtered technetium-99m sulfur colloid, the injection site clearance was faster after Lymphoseek administration which optimizes identification of the sentinel lymph node and reduces the radiation dose to the body *(18)*. These safety studies indicate that Lymphoseek has an acceptable radiation dose without exhibiting any biologically significant toxicities or pathologic abnormalities. The effects were examined by evaluating the overall toxicity, the histopathology, the cardiovascular pharmacology, and the perivascular irritation in several animal models (Table 1). The effective dose of Lymphoseek was determined to be half the value of the albumin-based nanocolloids *(21)*. When projecting this information to a patient with breast cancer, an administration of a 0.4-mCi dose to the breast would cause an estimated absorbed radiation dose to the injected breast of 0.22 rad (2.2 mGy). This would yield a radiation risk of 0.1 mSv which places sentinel lymph node mapping with Lymphoseek among other low-risk radiologic procedures, such as mammograms (0.4 mSv) and chest X-rays (0.02 mSv) *(21)*. Compared to other radiotracers, Lymphoseek therefore reduces the radiation exposure for both the patient and the surgical and the pathology teams.

Table 1
Preclinical Toxicity Studies for Lymphoseek

Test	*Species*	*Dose factor*[1]	*Result*
Acute toxicity	Rats and Rabbits	50 and 500	Subcutaneous injection of Lymphoseek at doses up to 7.0 nmol/kg in both species had no effect on survival, clinical observations, body weight, or gross and histopathology. In all treated female rabbits, eight out of ten treated male rabbits, and one control male rabbit, mild centrolobular hepatocytic hypertrophy was noted microscopically.
Acute toxicity	Dogs	170, 780, and 1700	Subcutaneous injection of Lymphoseek at doses up to 25 nmol/kg had no effect on mortality, clinical observations, body weight, food intake, or gross and histopathology. Mild inflammatory reaction was observed, consistent with a foreign material response by the host, rather than a direct toxic effect of Lymphoseek.
Perivascular irritation	Rabbits	100	Intramuscular injection of Lymphoseek at doses up to 14.3 nmol/kg had no significant clinical effects on survival, clinical observations including the injection site, or histopathology of the injection site.
Repeat dose[2]	Rats and Dogs	42, 85, and 170	Subcutaneous injection revealed no clinical abnormalities and no changes in body weight, food intake, physical and ophthalmic examination, urinalysis, or gross and histopathology with a Lymphoseek dose up to 2.5 nmol/kg per day, when consecutively administered for 14 days. No effects on hematologic and cardiovascular parameters that were additionally investigated in dogs were shown.
Cardiac safety pharmacology	Dogs	1700 and 3400	Intravenous injection of Lymphoseek dosed up to 50 nmol/kg had no effect on blood pressure, heart rate, EKG, body temperature, or plasma histamine and thromboxane B2 levels. There were also no effects noted on mortality, clinical observations, or body weight.
Sensitization	Guinea pigs	56, 113, and 1130	An intravenous injection of Lymphoseek dosed up to 17 nmol/kg did not induce any anaphylactic reactions in the animals. No effects were

(*Continued*)

Table 1
(Continued)

Test	*Species*	*Dose factor*[1]	*Result*
			observed on mortality, clinical and cage side observations, and body weight.
Lymphoma mutagenesis	Mice	300	In the lymphoma mutagenesis assay with or without Aroclor-induced rat liver S9 activation, no mutagenic potential could be shown for Lymphoseek.
In vitro reverse mutation[3]	Bacteria	0.3, 0.9, 3, 9, 90, and 300	Lymphoseek demonstrated to have no mutagenic potential, confirmed by a negative bacterial reverse mutation assay.
In vitro micronucleus	Mice	2.5, 5, and 10	Lymphoseek, at doses up to 0.12 mmol/kg, did not induce a significant increase in incidence of micronucleated polychromatic erythrocytes and was, therefore, concluded to be negative in the mouse micronucleus assay.

[1]times scaled human dose
[2]14 doses over 14 consecutive days
[3]nmol/ml

7. PHASE I AND PHASE II CLINICAL TRIALS

Lymphoseek's safety profile in patients was evaluated within several Phase I clinical trials, conducted in patients with breast cancer or melanoma. To determine the optimal dose of Lymphoseek for breast cancer patients, either filtered technetium-99m sulfur colloid or different doses of Lymphoseek (concentrations of 0.2, 1.0, or 5.0 nmol) were administered to 24 patients. These patients were divided into four groups consisting of six patients each who all received the same amount of radioactivity (0.5 mCi) *(26)*. The Lymphoseek dose of 1.0 nmol had the highest sentinel lymph node uptake per injected dose *(26)* and was, therefore, chosen for future clinical trials. This dose assured identification of all sentinel nodes without saturating their receptor sites, since that process had subsequently led to uptake in distal draining lymph nodes.

A study conducted in 12 breast cancer patients compared the injection site clearance and the sentinel node accumulation between Lymphoseek and filtered technetium-99m sulfur colloid, both injected with a peritumoral/subdermal technique *(27)*. Lymphoseek, with a clearance half-life of 2.72 ± 1.57 hours, was eliminated significantly faster ($p = 0.0025$) than technetium-99m sulfur colloid which exhibited a clearance half-life of 49.5 ± 38.5 hours. There was no statistically significant difference ($p = 0.75$) between the two agents in the sentinel node uptake at 3 hours postinjection. However, Lymphoseek demonstrated a higher concordance of sentinel node detection with isosulfan blue dye compared with filtered technetium-99m sulfur colloid *(27)*. A similar study in 10 breast cancer patients demonstrated a similar clearance half-life of 2.62 ± 0.55 hours for Lymphoseek after intradermal administration. This injection site clearance was significantly faster ($p = 0.001$) than that of filtered technetium-99m sulfur colloid with a half-life of 24.1 ± 17.7 hours. Again, both agents exhibited a statistically equivalent ($p = 0.28$) sentinel lymph node uptake *(28)*. These results were similar to a clinical study that addressed melanoma patients and investigated the injection site clearance, and sentinel lymph node accumulation of Lymphoseek in comparison with filtered technetium-99m sulfur colloid, also performing an intradermal injection *(29)*. Lymphoseek demonstrated a significantly faster ($p = 0.001$) clearance half-life of 2.17 ± 0.96 hours versus technetium-99m sulfur colloid with 14.7 ± 6.3 hours, and both radiopharmaceuticals exhibited a comparable ($p = 0.68$) sentinel lymph node accumulation *(29)*. These three studies confirmed that Lymphoseek was compatible with different injection techniques in sentinel node mapping and that Lymphoseek consistently exhibited a faster injection site clearance compared with filtered technetium-99m sulfur colloid. This quality of Lymphoseek reduced the injection site scatter in the lymphoscintigraphic image, and additionally, it diminished the radiation dose to the patient.

No adverse reactions related to Lymphoseek were reported in any of the patients participating in these clinical trials. All patients were monitored for clinically significant alterations in laboratory parameters, such as hematologic parameters, urinalysis, and serum chemistry panel. An estimated absorbed radiation dose calculated from clinical study data for breast cancer patients concluded that the radiation dose for Lymphoseek was 25% of the radiation dose of filtered technetium-99m sulfur colloid *(26)*. Using filtered technetium-99m sulfur colloid in melanoma patients, the remaining radiation dose after tumor excision was almost three times as high, compared with Lymphoseek *(29)*.

Figure 2 provides an example for sentinel node mapping via Lymphoseek. The lymphoscintigram was performed in a 50-year-old male patient who presented with a 1.04-mm melanoma on his left shoulder, identified as Clark level III, and negative for metastatic disease after histopathologic sentinel lymph node analysis. The images show an anterior (Panel A), a left lateral (Panel B), and a left anterior oblique view (Panel C) and were acquired 2 hours postintradermal injection of Lymphoseek (10 nmol injection and 0.5 mCi dose). One single sentinel node was identified by Lymphoseek and was removed 5.7 hours postinjection. It exhibited a gamma count of 1900 cps ex vivo (0.49% ID) with a background measurement of 20 cps. Additionally, this sentinel node was detected by isosulfan blue dye. Figure 3 shows the

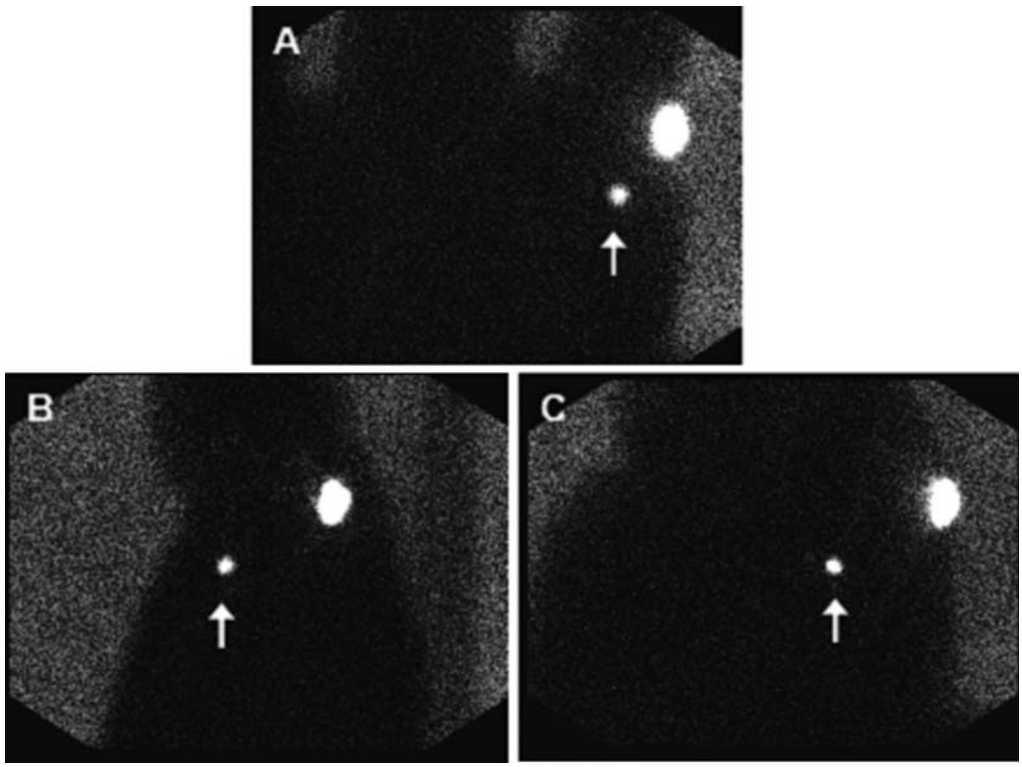

Fig. 2. Lymphoscintigram of a melanoma patient after lymphoseek injection. The lymphoscintigraphic images were obtained in an anterior (Panel **A**), a left lateral (Panel **B**), and a left anterior oblique view (Panel **C**), showing one single sentinel lymph node (*Arrow*) next to the injection site of Lymphoseek in a melanoma patient at 2 hours postintradermal injection.

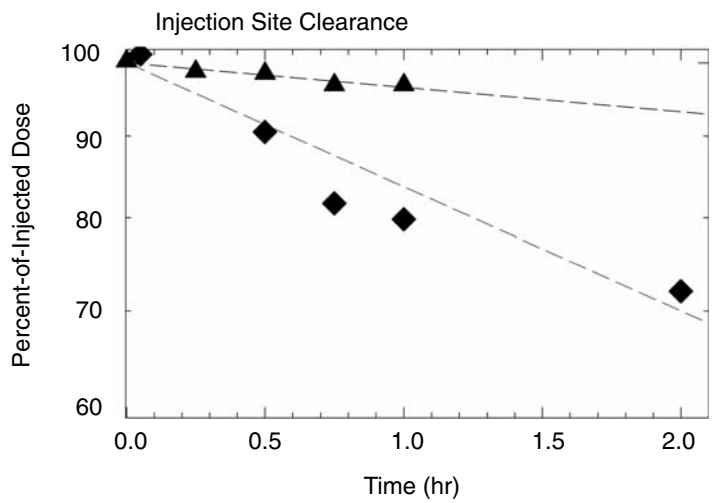

Fig. 3. Injection site clearance of lymphoseek versus filtered technetium-99m sulfur colloid in two melanoma patients. Lymphoseek (*Diamonds*) injected in a melanoma patient demonstrated a significantly faster injection site clearance half-life of 3.88 ± 0.65 hours compared with filtered technetium-99m sulfur colloid (*Triangles*) administered to a different melanoma patient and exhibiting an injection site clearance half-life of 19.7 ± 3.19 hours.

significantly faster Lymphoseek injection site clearance in the described subject (clearance half-life of 3.88 ± 0.65 hours) versus an injection site clearance of filtered technetium-99m sulfur colloid (clearance half-life of 19.7 ± 3.19 hours) in a different melanoma patient participating in this study.

After proving Lymphoseek's safety in patients with these Phase I clinical trials, a multicenter Phase II clinical trial was conducted to evaluate the effectiveness of Lymphoseek in breast cancer and melanoma patients *(30)*. The goal was to assess the applicability of Lymphoseek as a radiotracer for identification of the sentinel lymph node, using a gamma camera preoperatively and intraoperatively. The 80 patients participating in the Phase II study consisted of 31 breast cancer and 49 melanoma patients who all received 50 μg (3 nmol) of Lymphoseek injected in close proximity to the primary tumor. Results demonstrated a sentinel node identification rate of 95%, and only one sentinel lymph node not being detected by Lymphoseek was found to be positive for metastatic disease. Additionally, the concordance of Lymphoseek with blue dye was assessed, and it revealed a concordance rate of 89.5% for breast cancer patients and 97% for melanoma patients. Overall, adverse events were reported, but these events were unrelated to Lymphoseek.

8. CONCLUSION

Lymphoseek has demonstrated in both preclinical and clinical studies to be a versatile radiotracer that possesses better pharmacokinetic characteristics than the previous standard agent, filtered technetium-99m sulfur colloid. Clinical trials, in which Lymphoseek was successfully used for sentinel lymph node identification, have already confirmed these favorable properties of Lymphoseek in breast cancer and melanoma patients. Preclinical studies have also revealed promising results for the application of Lymphoseek in colon and prostate cancer patients. Additionally, Lymphoseek offers the possibility to be used in minimally invasive surgery as well, which is of increasing importance in surgery.

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13 Lymphatic Disorders in Patients with Cancer

Edwin C. Glass, MD and Emily Iker, MD

Contents

ABSTRACT

Lymphedema (LE) is a common complication in patients with cancer. LE results from an imbalance between the generation and the removal of interstitial fluid and macromolecules. Both congenital (primary) and acquired (secondary) LE demonstrate variability in their onset and severity. Although edema can result from any process causing an accumulation of interstitial fluid, LE refers to swelling resulting from lymphatic insufficiency. The onset of LE follows factors that trigger an imbalance in interstitial fluid dynamics. LE in cancer patients usually results from destruction of lymphatic tissue by surgery and/or radiation. When less extensive surgery and radiation are carefully used in the management of malignancies, the incidence and severity of LE appear to be reduced. Sentinel lymph node procedures can mitigate the incidence and severity of LE when complete lymph node dissections can be replaced by sentinel node sampling alone. It is important to clarify the diagnosis of LE in contrast to other conditions that may mimic it, such as lipedema, phleboedema, or heart failure. Many imaging modalities can demonstrate various features of LE including MRI, CT, and ultrasound of the epifascial

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compartment, which is the tissue most commonly involved with LE. Lymphatic function and lymph drainage are readily evaluated with lymphoscintigraphy (LS). Lymphatic imaging plays an important role in diagnosing, classifying, and determining the severity of swollen extremities and in predicting the success of common therapies. LE is a chronic and treatable disorder, and early recognition and treatment should be promulgated.

Key Words: lymphedema; complications of lymphadenectomy; lymphoscintigraphy; lymphatic kinetics; interstitial fluid

Lymphedema (LE) is a common complication in patients with cancer. Lymphedema results from an imbalance between the generation and the removal of interstitial fluid and proteins. Swelling results when the lymphatic system fails to remove interstitial fluid at the rate it is accumulating.

1. DEFINING LYMPHEDEMA

Although LE can ensue from any imbalance between the generation and the removal of interstitial fluid and protein, the term lymphedema refers to edema caused by lymphatic insufficiency, which results in inadequate removal of fluid and macromolecules. Common causes of lymphatic insufficiency include surgical excision or ligation of lymphatics, radiation fibrosis, lymphatic obstruction or obliteration (e.g., due to filariasis or tumor invasion), congenital hypoplasia of lymphatics, extrinsic lymphatic compression by cysts or tumors, or dysfunction of other causes. With reduced lymph flow, proteins and macromolecules accumulate in the interstitial space, resulting in greater oncotic retention of fluid. Furthermore, these retained waste products engender and aggravate chronic inflammation, with eventual tissue fibrosis. Inflammation and fibrosis further constrict and impair the egress of fluid through easily collapsible, thin-walled lymphatic vessels. Hence the process tends to progress if not interrupted. This evolution of LE is incorporated and characterized in the clinical staging system proposed by the International Society of Lymphology (ISL) *(1)*, (Table 1), as well as in other staging systems.

LE can cause gross disfigurement and significant debilitation. It limits mobility of the limb, and can be painful. The affected extremity is prone to infections, often requiring hospitalization. If not treated, the disease progresses and can cause elephantiasis of the limb. The psychological

Table 1
Staging of Lymphedema: International Society of Lymphology (ISL)

Stage 0
Preclinical state. Lymph transport is impaired although swelling is not evident. May exist months or years before onset of clinical edema.
Stage I
Early onset of edema. Swelling subsides with elevation. Edema may be pitting.
Stage II
Pitting present, no response to elevation, fibrosis in later stages.
Stage III
Tissue hard and fibrotic. Does not pit. Trophic skin changes develop including thickening, hyperpigmentation, and hyperkeratosis.

impact can also be severe. Since the process is often multifactorial, it is seen commonly in patients with other diseases and pathophysiology including heart disease, cancer, obesity, and immobilization, among others.

2. PATHOGENESIS OF EDEMA INCLUDING LYMPHEDEMA

Lymphatic circulatory systems were operative in primitive organisms, long before the evolution of hemodynamically driven circulatory systems. These initial lymphatic circulations provided a mechanism for the delivery of nutrients and the removal of waste substances in multicellular organisms *(2)*. While the former task of delivery has been superseded by more efficient circulatory systems in higher organisms, the latter task of removal is still handled at least in part by the slower moving lymphatic circulatory system.

The interstitial space is both extravascular and extracellular. It contains the majority of the body's albumin pool *(3)*. Since the volume of this space cannot be measured anatomically, dilutional methods are utilized to assess its volume. These indicate that it has a volume approximately twice that of the blood volume *(4,5)*. Large molecules, proteinaceous aggregates, and cellular debris accumulate in this space as senescent cells degenerate and slough membrane fragments, and as bacteria or other foreign materials accumulate. Many of these macromolecules and particles are too large to be absorbed through capillary or venous walls for removal by circulating plasma. Instead, the lymphatic system is essential for removal of these waste products.

How are they removed? The distal ends of lymphatics contain clefts in their walls that communicate with the interstitial space, allowing a route of egress for interstitial fluid, particles, and macromolecules. The clefts open and close under the influence of relative hydrostatic and oncotic pressures in the lumen of the lymphatic versus that of the interstitial space as diagrammed in Fig. 1. Additionally, macrophages and other scavenger cells can carry engulfed particles and adherent molecules through the walls of the lymphatic vessels and through these clefts, for subsequent downstream delivery to, and processing within, afferent lymph nodes.

At the tissue level, at least three compartments interact with each other for the regulation of regional fluid volumes: the vascular space, the interstitial space, and the lymphatic space. Starling forces of both hydrostatic and oncotic pressure modulate the movement of fluid between these spaces and within each of the spaces. Since vascular fluid is rapidly transported away, the volumes within the lymphatic space and especially the interstitial space are the principal sites where excess fluid or edema accumulates. As hydrostatic pressure increases within the vascular compartment, fluid accumulates in the interstitial space, as occurs for example with venous obstruction, venous valvular insufficiency, or heart failure. These processes result in increased

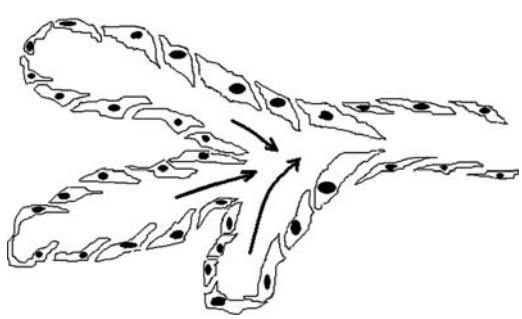

Fig. 1. Clefts in walls of distal lymphatic allow exchange of fluid and macromolecules between interstitial space and lymph vessel.

hydrostatic pressure within, and increased egress of fluid from, small vessels and capillaries. As oncotic pressure increases in the interstitial space, such as with inflammation and the retention of macromolecules in chronic edema, fluid is drawn into and retained within the interstitial space. Enhanced capillary permeability due to inflammation or other causes also results in an increased interstitial fluid load requiring lymphatic removal. Edema develops whenever the lymphatic system is unable to remove this increased flux of fluid.

Alternatively, if hydrostatic pressure increases within the lymphatic vessels due to lymphatic valvular insufficiency, an inadequate number of lymphatic vessels, or lymphatic obstruction, then the removal of interstitial fluid will not match the rate of fluid accumulation, which also results in edema. In sum, edema results whenever the input exceeds the output of fluid in the interstitial space.

At any given level of compromise of lymphatic function, any process generating additional interstitial fluid will aggravate edema. Examples include heart failure, increased venous pressure due to phlebitis or postphlebitic syndrome, inflammation due to cellulitis, paralysis, or prolonged dependency; these can all worsen, or alternatively may precipitate edema in a predisposed individual with compromised lymphatic capability. The central role of fluid volume is reflected in the grading system proposed by the ISL, ranking severity of unilateral LE of a limb as mild (<20% excess limb volume), moderate (20–40% excess), and severe (>40% excess) *(1)*. Although changes in interstitial fluid volume are central to the pathogenesis of LE, the syndrome is complex and often progressive, as discussed above. Hence, other grading systems incorporate criteria other than volume change alone in the assessment of LE, including dermal and soft tissue changes *(6)*.

3. BROAD CLASSIFICATION OF APPENDICULAR LYMPHEDEMA

Recognizing that LE usually refers to edema that arises from compromised lymphatic transport, it is apparent that the process may develop in any part of the body. Most often, however, it is manifested as edema of an extremity. LE of the limbs is broadly classified as primary, due to an innate lymphatic insufficiency, or secondary, resulting from an acquired derangement of lymphatic structure or function. Mixed forms occur, however, since any individual with an inborn deficiency of lymphatic structures will be predisposed to develop LE with relatively milder insults to their lymphatic system than would an individual with more generous lymphatics.

4. PRIMARY LYMPHEDEMA

Primary LE results from a congenital deficiency or absence (aplasia) of lymph structures, including lymph nodes and/or lymph vessels. Most commonly, primary LE becomes manifest as swelling of in one or both lower extremities, usually in an asymmetric pattern (Figs. 2 and 4).

Primary LE is classified as true congenital LE when present at birth (in ~1/6,000 births) or when it has an onset before 1 year of age. Although usually nonfamilial, a rare familial form is Milroy's disease *(7)*, associated with the VEGFR-3 gene mutation. SOX18 gene mutations result in the hypotrichosis–lymphedema–telangiectasis syndrome *(8)* (Fig. 3).

More commonly primary LE first becomes clinically evident before the age of 35 of age. It is then classified as LE praecox. This presentation occurs in ~60–75% of cases of primary LE. It begins as a unilateral swelling in the distal part of lower extremity affecting the ankle, dorsum of the foot, and toes, more commonly involving the left leg (~2:1 ratio of left to right in our experience). The disease is more prevalent in females (Fig. 4). Patients frequently

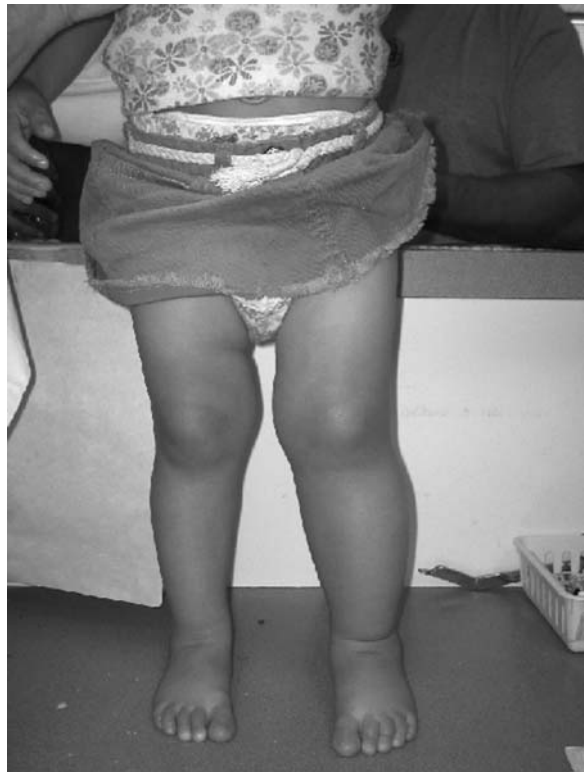

Fig. 2. Congenital lymphedema (LE) in a child with onset in infancy.

report a history of sudden onset, triggered by an event that results in an overload of the lymphatic transport capacity of the leg. Examples of trigger events we have witnessed include minor surgery, insect bites, cat scratches, jellyfish stings, infections, trauma, prolonged travel, and other events that escalate a generation of interstitial fluid that exceeds the drainage capacity in a leg that is deficient in lymphatic functional capacity. Once initiated, the edema often then continues as a chronic disorder due to the pathophysiologic processes discussed above.

Physical findings in patients with primary LE of the lower extremities include (1) Stemmer's sign (difficulty in lifting skin on dorsum of toes or fingers due to thickening and induration.), (2) thickening of skin folds, (3) swelling of dorsal aspect of foot, (4) retromalleolar swelling, and (5) swelling of medial aspect of the knee.

An innate deficiency of lymphatics may sometimes become evident later in adult life. When the onset occurs after the age of 35, it is classified as LE tarda or Meige's disease.

The various forms of primary LE discussed above result from an inborn deficiency of lymphatic structures. Lymphedema results from reduced lymphatic transport capacity and accumulation of interstitial fluid and proteins. Therefore LE patients are at higher risk of postoperative complications. Although most people have a normal lymphatic capacity as demonstrated in the right panel of Fig. 4, those with lymphatic deficiencies vary widely in severity, as demonstrated in Figure 5. The surgical removal of even a single lymph node from a person with a deficient endowment of lymphatics can result in severe LE.

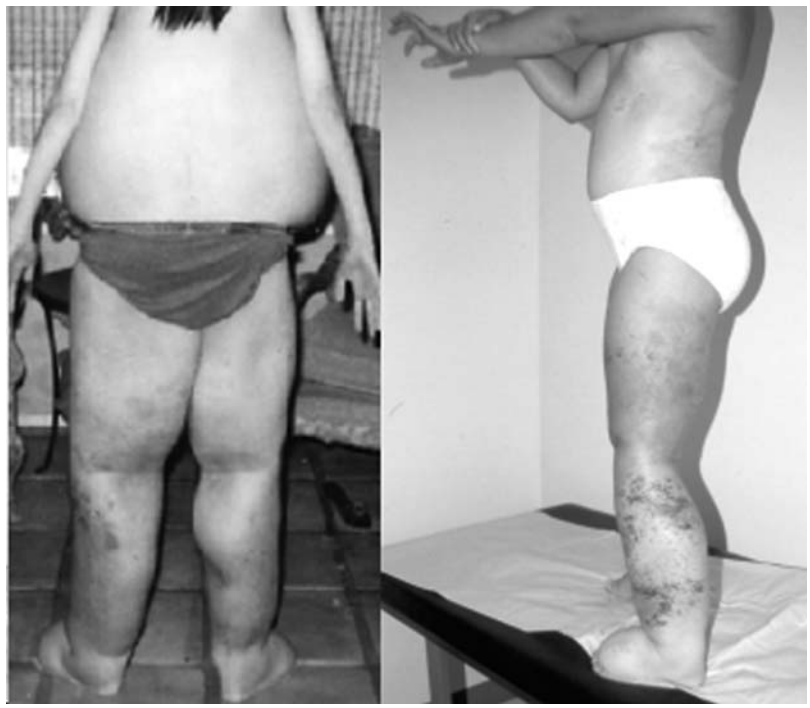

Fig. 3. Hypotrichosis–lymphedema–telangiectasis syndrome. Both LE and cutaneous port wine telangiectases are present in the lower extremities of this young girl with Klippel–Trenaunay Syndrome.

5. SECONDARY LYMPHEDEMA

Secondary or acquired LE results from processes that disrupt the balance between the generation and the removal of interstitial fluid. In the case of venous edema, elevated venous and capillary pressures resulting from venous obstruction or venous valvular insufficiency result in excess generation of fluid. In these individuals, we and others have observed accelerated lymphatic flow in the affected extremity using lymphoscintigraphy (LS) *(9)* (Fig. 6). Combined insufficiency of venous and lymphatic systems leads to phlebolymphedema, commonly seen in patients after saphenous vein ligation and stripping for coronary bypass grafting.

Worldwide, the most common cause of [secondary] LE is filariasis, principally caused by Wucheria Bancrofti, Brugia Malayi, or Brugia Timori. Mosquitoes ingest microfilariae from an infected individual. The larvae develop in the mosquito and are subsequently transmitted to other humans by the mosquito vectors. Within the second host the larvae develop into adult worms that localize in lymphatics *(10)*. Local inflammation and subsequent fibrosis result in lymphatic obstruction and LE. Although antifilarial drugs can kill the offending filarial organisms, the pathophysiologic processes discussed above in Section 2 often persist, resulting in progressive and severe disability *(10,11)* (Fig. 7).

In more developed countries, secondary LE most often results from surgery and/or radiation (Figs. 8, 9, 11, 12). This occurs most commonly in the treatment of malignancies with lymphatic invasion. Therefore the required treatment will remove or destroy the disease as well as interrupt the regional lymphatic structure and function. Such iatrogenic LE is seen most commonly in the treatment of breast cancer, melanoma, pelvic and genital malignancies, head and neck cancer,

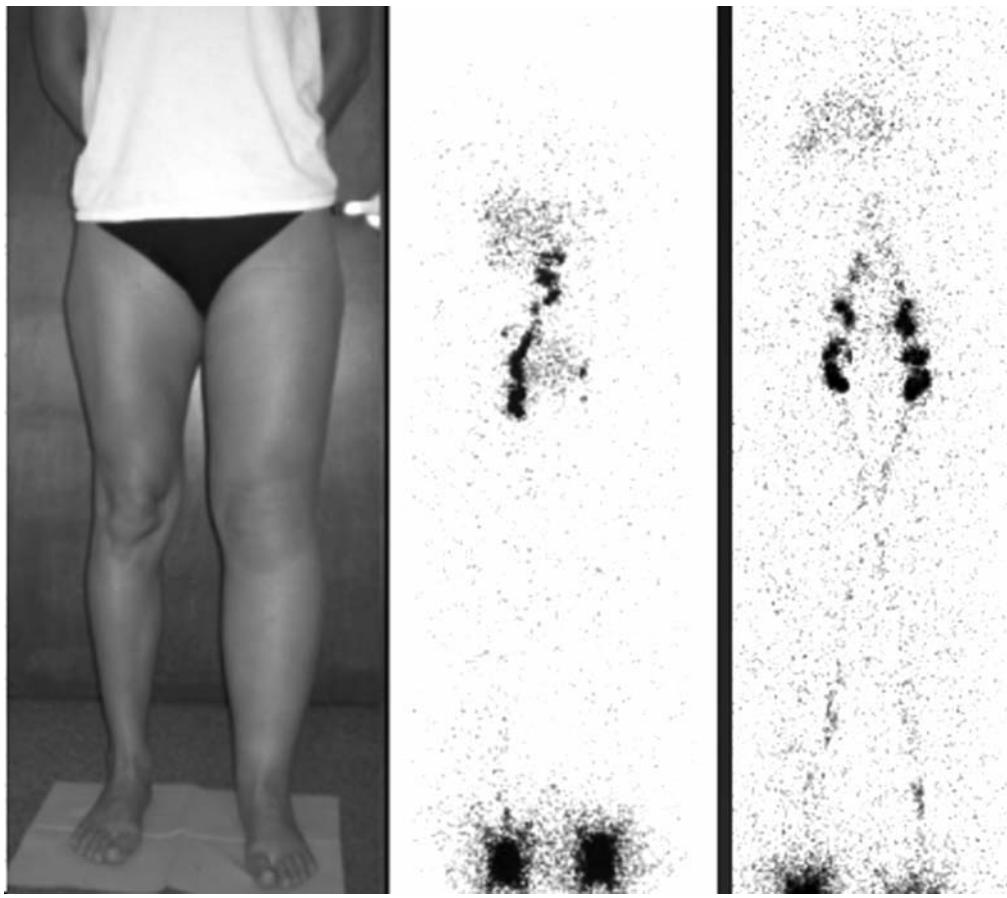

Fig. 4. LE Praecox. This 24-year-old woman reported a recent and sudden onset of swelling of her left leg after an insect bite followed by mild local cellulitis. Her lymphoscintigram (*middle panel*) demonstrates fairly normal lymphatic function in right leg, but deficient, although not completely absent, lymphatic structures and function in the left leg. *Right panel* demonstrates a normal lymphoscintigram from a different individual for comparison.

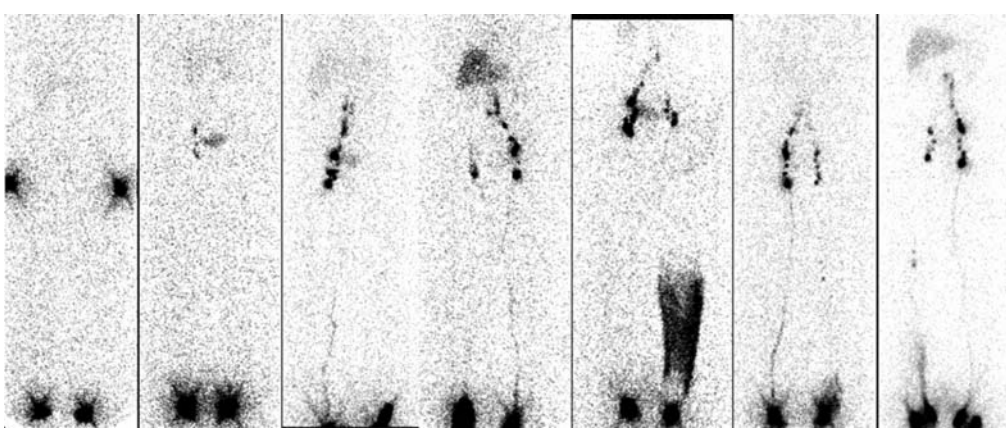

Fig. 5. Variability of lymphatic capacity in primary LE: A set of lymphoscintigrams from seven individuals with primary LE. Lymphoscintigram on far left demonstrates almost complete absence of lymphatic nodal structures, but moving rightward, other individuals demonstrate sequentially greater lymphatic integrity. Study on far right demonstrates an asymmetric, but almost normal pattern.

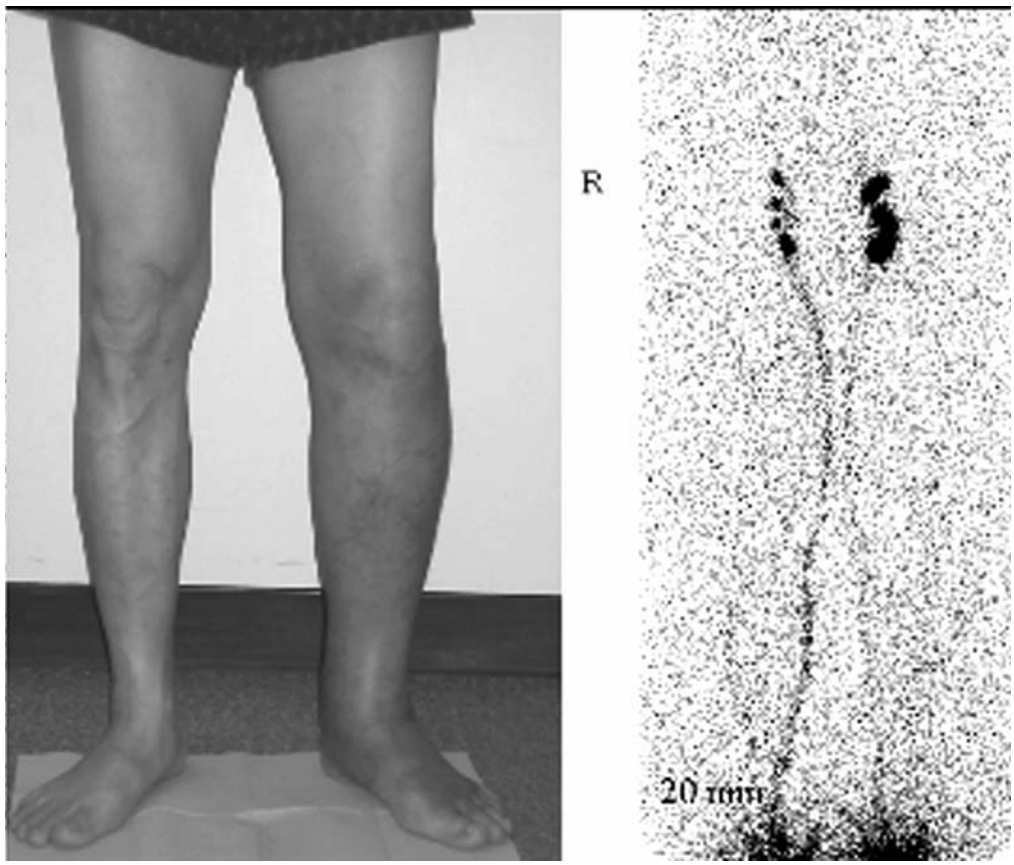

Fig. 6. Sixty-two-year-old male with chronic swelling of left leg, previously diagnosed as primary LE. Lymphoscintigraphy (right) demonstrated intact lymphatic structures in both legs, but with accelerated lymphatic flow in affected extremity (note greater accumulation of tracer in left inguinal nodes than in right). Doppler studies subsequently confirmed venous insufficiency in left leg.

and soft tissue sarcomas. Regional nodal sampling, lymphadenectomy, and postoperative radiation are integral to the management of these malignancies for staging, prognostication, control of locoregional disease, and possibly for prolonging survival.

6. LYMPHEDEMA AND BREAST CANCER

A large number of investigations have addressed the problem of LE after treatments for breast cancer. Prior to the use of sentinel node techniques, LE after axillary lymphadenectomy for breast cancer was reported to occur in 10–49% of patients *(12–18)* (Fig. 8). Many investigations have addressed and identified factors associated with its occurrence, although considerable variation in the incidence of the problem is apparent. This wide variation relates to different definitions of what constitutes LE, varying the extent of surgery, different methods of measurement, variable duration of follow-up, as well as to variations in the prevalence of predisposing factors in the populations studied *(6)*.

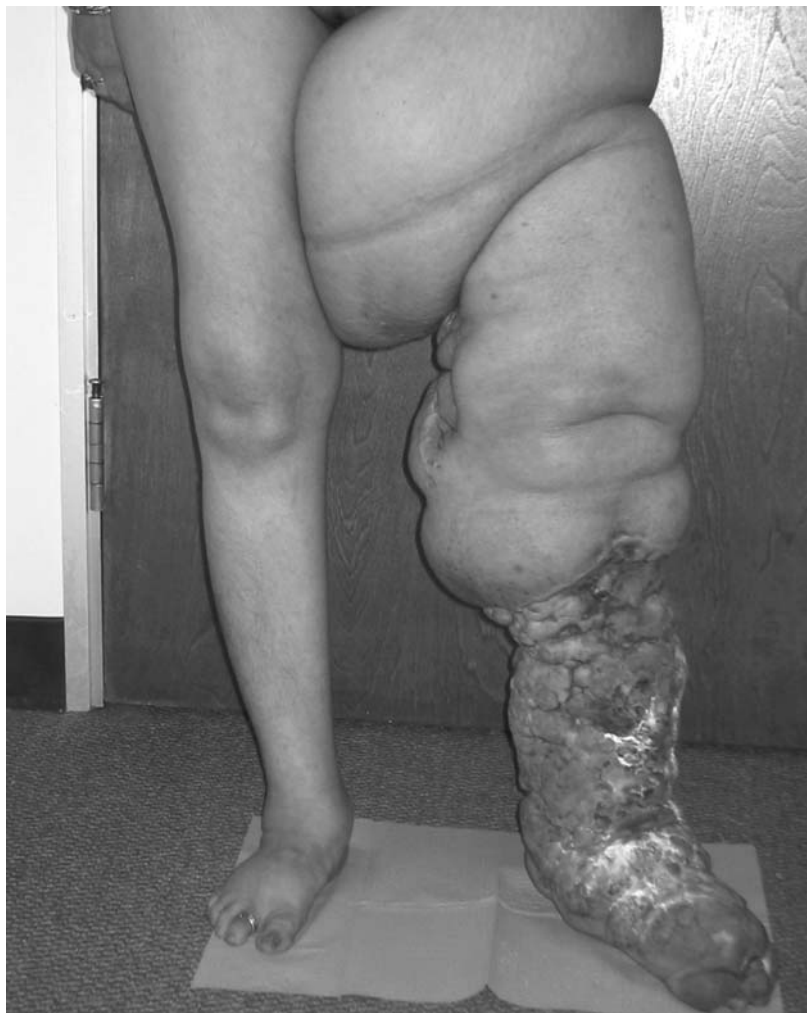

Fig. 7. Fifty-one-year-old woman from India with 25-year history of filariasis of the left leg.

Among the definitions of LE of the arm employed are: an increase in volume of >200 ml *(19,20)*, an increase in circumference of more than 2.5 cm *(21)*, an increase in circumference of more than 1.5 cm at two or more sites *(22)*, or an increase in more than 2 cm at one location *(23)*.

Among the methods of measurement employed are subjective clinical impression *(24,25)*, measurement of circumference of arm at a single location *(21,23)*, measurement of arm circumference at multiple locations *(22)*, water displacement *(19,20)*, and self-reported incidence *(23,26)*. Still, good correlations have been reported between circumferential measurements and the gold standard, water displacement *(27)*. Efforts have recently been directed toward standardizing both the definition and the assessment of LE in clinical trials *(6)*.

Variable durations of follow-up employed in different studies undoubtedly affect reported incidences as well. Meric reported a median delay to onset of 17 months *(28)*, and Powell et al. *(29)* reported a median delay of 39 months. We have observed patients who reported the onset of the disease over 5 years after treatment.

Many individuals who do not manifest apparent swelling (Stage 0, Table 1) may later develop overt disease after a triggering insult that results in the generation of an increased interstitial fluid

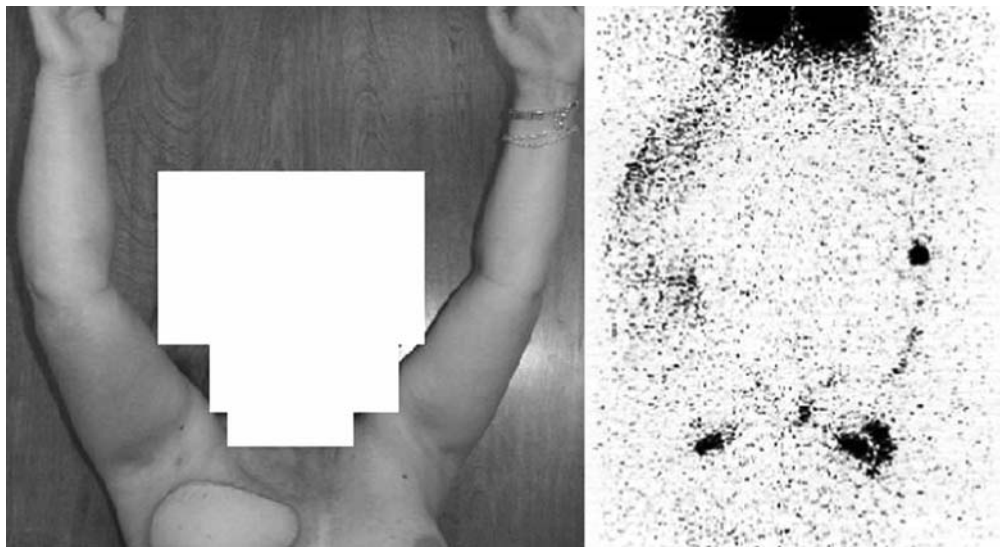

Fig. 8. LE of right arm after mastectomy, axillary lymphadenectomy, and irradiation for breast cancer. *Right Panel*: Lymphoscintigram demonstrates dermal backflow and retention even though some right axillary nodes are still present.

load in the affected extremity, such as trauma, phlebitis, or infection. Patients may not voluntarily report to their health care professionals new, but mild symptoms of tension, pressure, and heaviness in the breast or arm, particularly if they are not informed to look for these signs. Still, early intervention with manual lymphatic drainage, compression garments, and other therapies can ameliorate the course of the disease. It is important, therefore, that patients be informed about signs and symptoms and that they have careful follow-up.

Risk factors for developing LE of the arm and breast include increased body mass index *(28–33)*, more extensive surgery (which a priori is often associated with more extensive disease) *(33–36)*, axillary dissection *(20,28,37,38)*, removal of greater numbers of axillary nodes *(39,40)*, postoperative radiotherapy (particularly to the supraclavicular region) *(32,35,36,41,42)*, and infection *(17,43)*. The syndrome arises after axillary dissection for causes other than breast cancer as well (Fig. 9).

7. LYMPHEDEMA AFTER SENTINEL NODE SURGERY FOR BREAST CANCER

The use of sentinel node procedures has reduced the overall incidence of complications in the surgical treatment of breast cancer *(13–15,23,44–46)* and melanoma *(47,48)*. Prior to the use of sentinel node techniques, the incidence of LE after axillary node dissection for breast cancer has been reported as 10–49% *(12–18)*. More recent reports indicate an incidence of at least 6–7% with sentinel node biopsy alone, without axillary lymph node dissection *(23,46,49)*. Although the removal of sentinel nodes for breast cancer is intended to address only nodes that drain the breast, there is much commonality of nodal afferent input from the breast and the arm.

It may seem intuitive that the less extensive dissections employed for sentinel node sampling would be associated with a reduced incidence of LE compared with complete axillary dissection and lymphadenectomy, and indeed, some investigations have found lower rates of LE with sentinel node-sampling alone *(42,44–46,50)*.

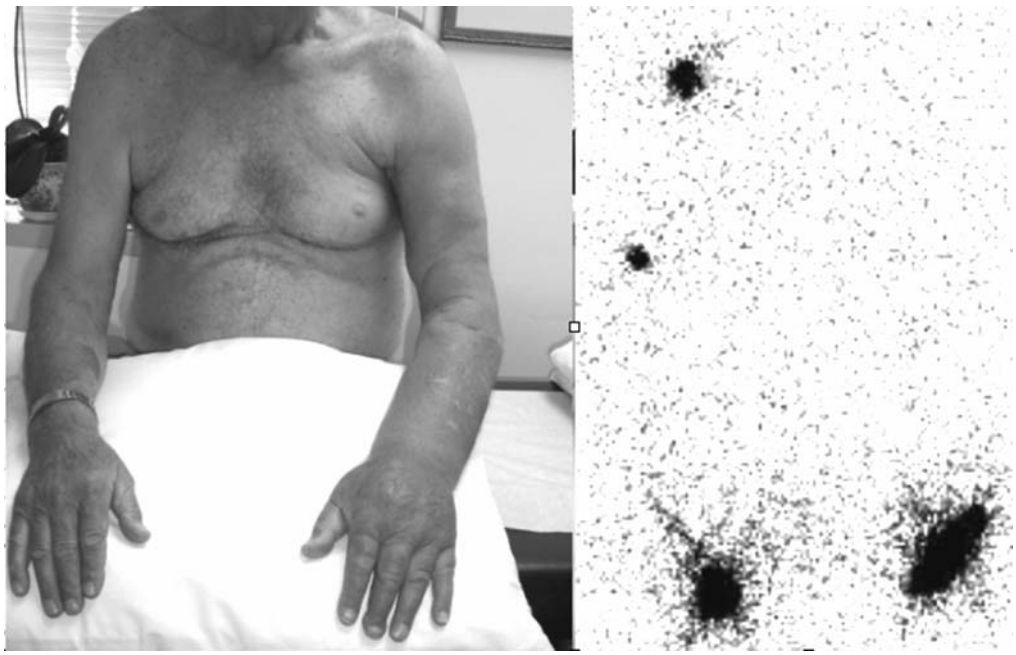

Fig. 9. This 78-year-oldman developed swelling in his left arm after axillary dissection for Merkel cell cancer. Lymphoscintigraphy (*right*) demonstrated absent lymphatic migration at 2 hours after injection on dorsum of left hand.

However, other investigations have not confirmed a lower incidence *(19,51,52)*. The overall treatment of breast cancer involves more than axillary surgery alone, and most patients receive breast irradiation regardless of other treatments. In the American College of Surgeons Z0011 trial involving only patients with positive sentinel nodes, the incidence of LE by circumferential arm measurement at 1 year was not significantly different among those who underwent complete axillary dissection than among those who underwent sentinel node removal alone, even though self-reported incidences were significantly different *(23)*. However, as discussed above, many patients first develop LE years after treatment. More protracted follow-up of patients treated with sentinel node surgery alone should provide an improved understanding of the impact of the procedure on the crucial questions of survival and rates of long-term complication, including LE.

Innate lymphatic capacity is variable in different individuals (Fig. 5), and hence patients who have congenitally limited numbers of lymphatic structures (nodes and lymphatic vessels) are more susceptible to developing the complication for any given level of lymphatic compromise resulting from treatments they receive.

8. LYMPHEDEMA AFTER INGUINAL NODE SURGERY

The legs are as susceptible if not more susceptible to the development of LE than the arms following lymphadenectomy. In the Sunbelt Melanoma Trial LE was found to be more common in the groin than in the axilla after either sentinel node biopsy alone (1.5 vs. 0.3%, respectively) or after complete node dissection (31.5 vs. 4.6%, respectively) *(53)*. Similarly, an investigation by Serpell et al. *(54)* disclosed an incidence of 29% following complete groin dissection compared with 6% after complete axillary dissection in 64 patients with melanoma. An overall incidence of

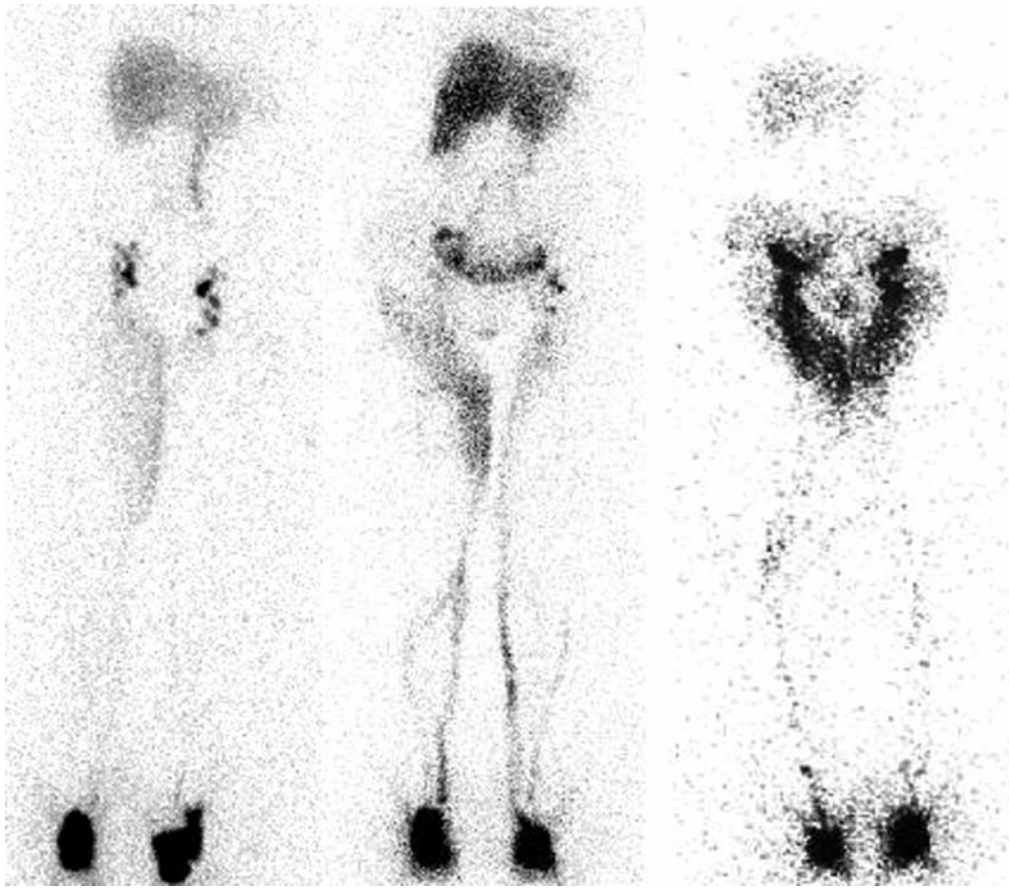

Fig. 10. Lymphoscitigraphic findings in three examples of LE in the anteromedial aspects of the thighs, an area that is particularly susceptible to the development of this complication. Following epifascial pedal injections, tracer retention/delayed clearance is demonstrated in lymphedematous areas of the thighs. *Left Panel*: Limited and predominantly unilateral involvement. *Middle*: Asymmetrical involvement. *Right*: Bilateral involvement.

21% was reported by Karakousis et al. *(55)* after complete groin dissection. Predisposing factors for LE after inguinal surgery are similar to those after axillary dissection, including increased body mass index, postoperative seroma, infection, more extensive surgery, and postoperative radiation *(48,54,56)*.

We have frequently observed edema localized to the anteromedial aspect of the thigh in particular, even in patients who do not manifest significant edema in the remainder of the affected leg (Figs. 10 and 18). In 1977, Jackson et al. *(57)* termed this finding, as demonstrated by LS, the scintigraphic "flare sign" (Fig. 10). Studies by Urist et al. *(56)* and Karakousis et al. *(58)* similarly emphasized the frequent occurrence of edema in this location.

9. LYMPHEDEMA AND GENITOURINARY CANCER

Penile and vulvar malignancies demonstrate lymphatic drainage and metastatic spread to the iliofemoral nodes, and therapy therefore involves groin surgery. Indeed, the concept of sentinel node mapping and lymphatic drainage was first reported for penile cancer by Cabanas *(59)*. LE

following inguinal node dissection for penile carcinoma has been reported in a variable percentage of cases, ranging from 16 to 100% *(60–62)*. In the treatment of vulvar carcinoma, incidences of LE of 25–30% have been reported *(63,64)*.

Malignancies deeper in the pelvis demonstrate variable lymphatic drainage, externally to the groin and/or internally through internal iliac, obturator, and common iliac lymph nodes and channels. LE following the treatment of these malignancies will reflect the lymphatic networks, basins, and channels affected and may become manifest as edema of the entire leg (Figs. 11 and 12), the anteromedial thigh (Fig. 10), or localized to the pelvis (Fig. 13). LE following the treatment of cervical cancer has been reported in 20–25% of cases *(65,66)*, although other studies with longer follow-up periods have found incidences as high as 49% by 10 years *(67)*.

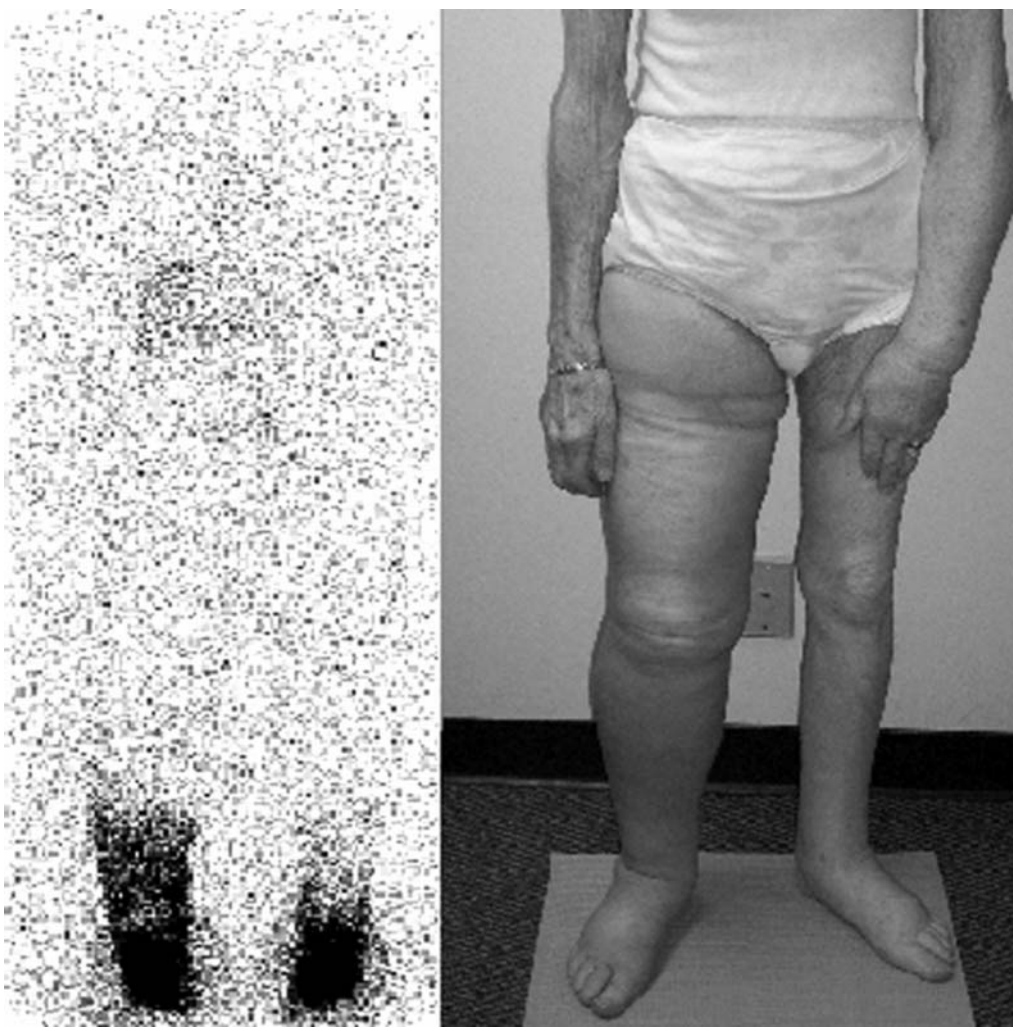

Fig. 11. *Right*: Chronic LE of right leg and left arm after pelvic and inguinal lymphadenectomy for uterine cancer and left axillary dissection for breast cancer, respectively. *Left*: Lymphoscintigram of lower extremities demonstrates poor overall lymphatic transit, with dermal backflow and retention, demonstrated commonly with ISL Stage II and III disease (Table 1).

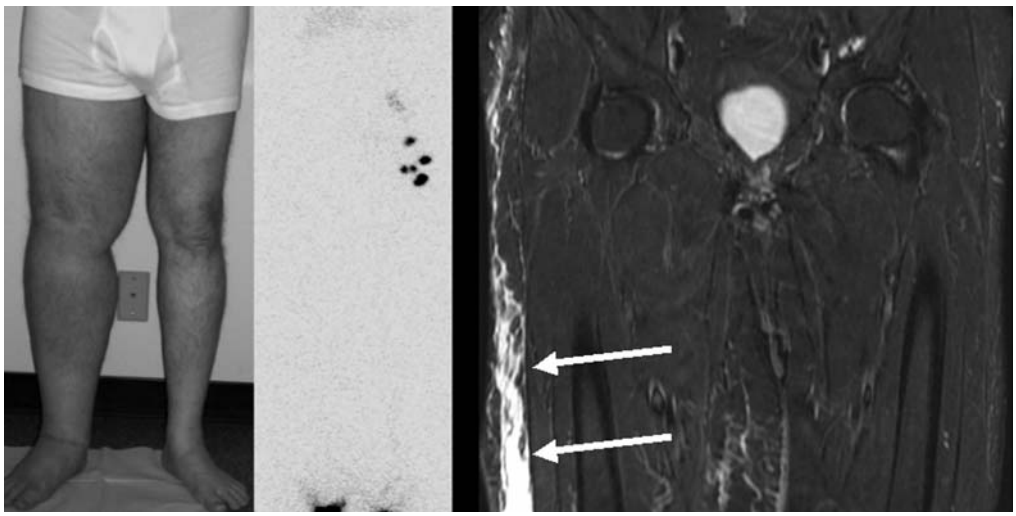

Fig. 12. Secondary LE after surgery for bladder cancer. *Middle Panel*: Lymphoscintigram demonstrates no lymphatic flow in edematous right leg 2 hours after injection in both feet, with normal migration to left inguinal nodes. *Right Panel*: Fat-suppressed magnetic resonance image demonstrates edema in epifascial compartment (*arrows*).

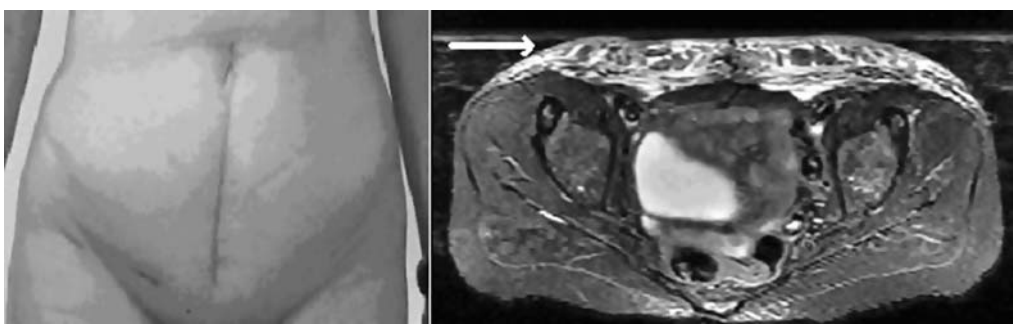

Fig. 13. *Left Panel*: Regional epifascial edema of anterior pelvis after regional lymphadenectomy for vaginal carcinoma, without LE of the lower extremities. *Right Panel*: Axial fat-suppressed inversion recovery MRI image of pelvis of same patient demonstrating anterior edema (*Arrow*).

The likelihood of development or recognition of LE after surgery for pelvic malignancies, as with other malignancies, is related to duration of follow-up, the use of adjuvant radiotherapy, the extent of surgery, and other factors discussed above for axillary and inguinal surgery.

10. LYMPHEDEMA AND SARCOMAS

Sarcomas often occur in the extremities. They frequently are bulky tumors that may cause primary lymphatic invasion as well as secondary compression of lymphatic vessels (Fig. 14). Surgical excision using wide local margins is a mainstay in their management, which may directly or indirectly destroy or compromise lymphatic structures. Consequently, secondary LE is commonly observed in these patients.

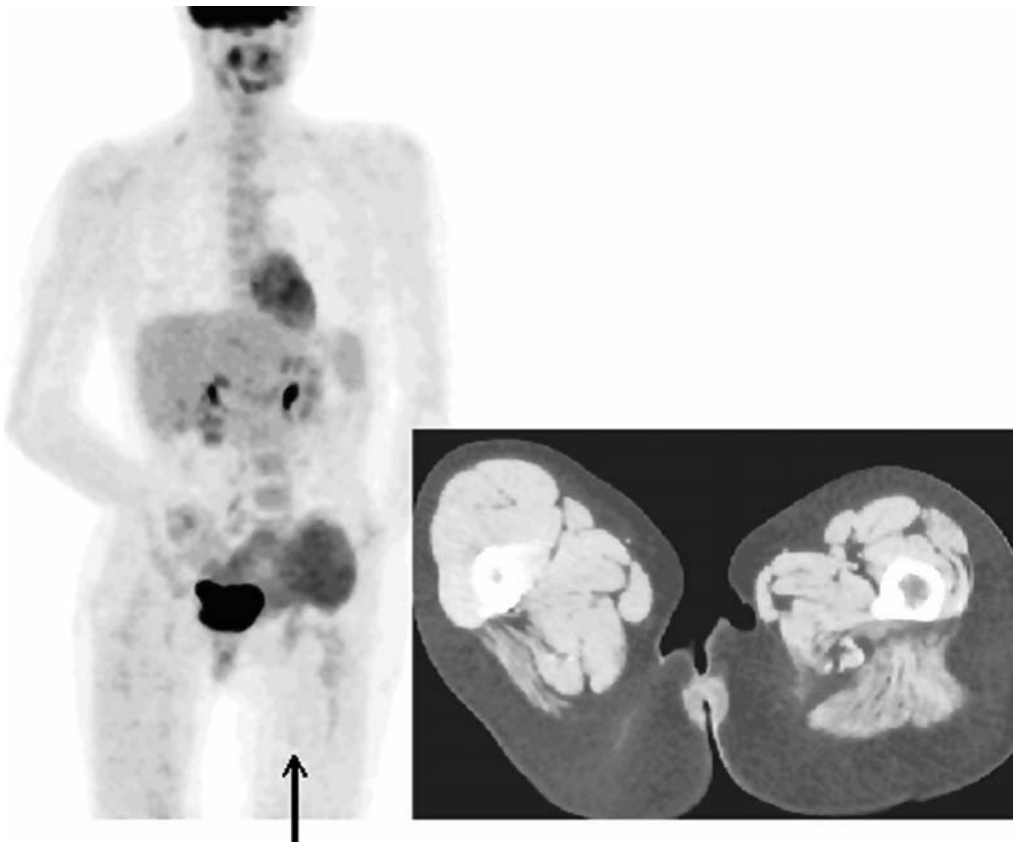

Fig. 14. Left Panel: PET scan demonstrates large left pelvic sarcoma, with secondary edema of left leg (*arrow*), demonstrated in both PET image (*left panel*) and also in CT sections of thighs (*lower right panel*).

Conversely, chronic LE is also associated, although infrequently, with the development of secondary angiosarcomas (Fig. 15). Secondary angiosarcoma is a malignant tumor of vascular origin, seen more commonly after breast cancer surgery and radiation *(68)* (Fig. 15). Less commonly it affects a lymphedematous lower extremity. The prognosis is poor even with the surgical excision, radiation, and/or chemotherapy.

11. OTHER CAUSES OF SECONDARY LYMPHEDEMA

Other causes of secondary LE are less common than those discussed above, but any process resulting in impaired lymph drainage of an extremity can result in LE. Examples include lymphatic invasion by metastatic malignancy (Fig. 16), sarcoid, burns, trauma, untreated pelvic malignancies, infection/cellulitis (Fig. 17), lymphomas, soft tissue sarcomas, synovial cysts of the knee or hip (Fig. 18), saphenous vein stripping for coronary bypass, or liposuction procedures.

12. DIAGNOSTIC IMAGING IN LYMPHEDEMA

All the common imaging modalities have been applied to the evaluation of patients with possible or known LE. Computed tomography (CT) clearly depicts anatomy and hence detects gross structural or mechanical causes of lymphatic obstruction, such as synovial cysts (Fig. 18) or tumors of the pelvis. Magnetic resonance imaging has many uses in

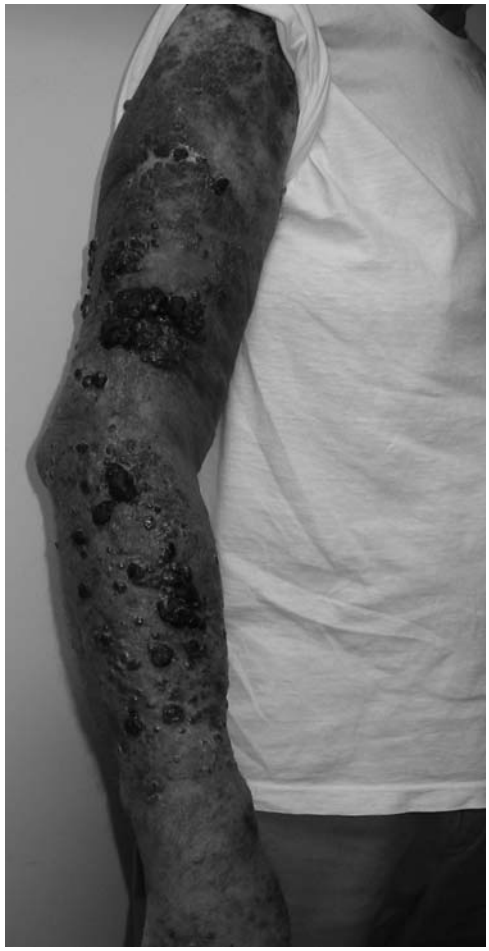

Fig.15. Angiosarcoma that developed in arm of patient with chronic LE.

patients with edema, including localization of LE to subfascial or epifascial compartments (Figs. 12, 13), demonstration of enlarged lymph vessels, and identification of structural causes of edema as with CT *(69)*. Ultrasound is useful for the evaluation for deep venous thrombosis, determination of tissue consistency, and measurement of epifascial depth as a quantitative index of edema (Fig. 19). Oil contrast lymphography is still utilized occasionally when necessary for identification and demonstration of lymphatic channels, such as in preoperative planning.

Other nonimaging methods applied to the diagnosis and evaluation of LE include the measurement of electrical impedance in tissue *(70,71)* and the measurement of biomechanical characteristics of tissue *(72,73)*.

13. LYMPHOSCINTIGRAPHY

LS is effective in demonstrating the patterns of this imbalance. Although widely employed for sentinel node localization, LS is also very useful in studying LE and other lymphatic disorders.

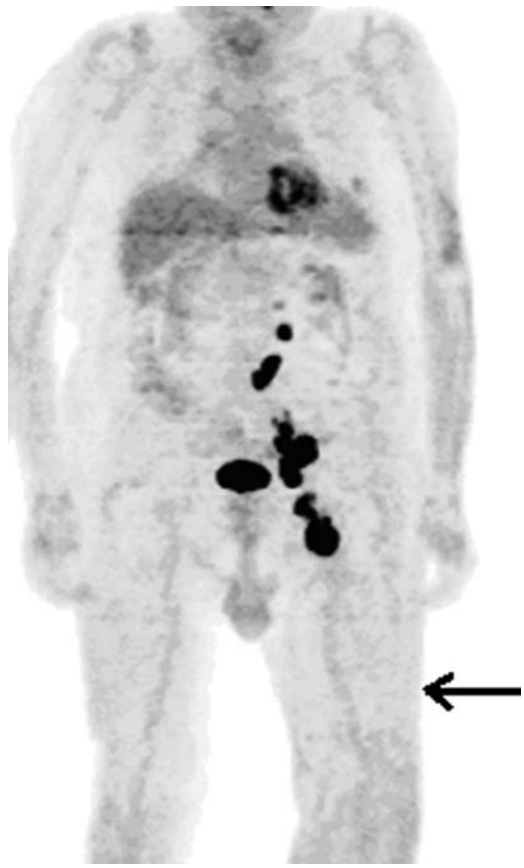

Fig. 16. FDG PET scan demonstrating metastatic melanoma to left inguinal, external iliac, and retroperitoneal nodes and lymph channels that has caused impedance of lymphatic flow and secondary LE in left leg (*arrow*).

LS is widely utilized in the functional evaluation of the lymphatic system. In addition to its well-recognized use in sentinel node localization, it is also commonly employed to clarify the diagnosis or cause of limb swelling. Examples include differentiation of primary and secondary LE (Figs. 4, 6, 8–10, and 12), differentiation of lipedema and LE, differentiation of primary LE and venous edema (Fig. 6), determining lymphatic capacity in patients with LE (Figs. 4–6, 11, 12, and 18), evaluation of chylous disorders *(74)*, and clarification of routes of lymphatic flow.

The technique for whole-body LS consists of explanation of the procedure to the patient, sterile preparation of the skin, local anesthetic in the dorsal aspect or interdigital space of the foot or hand of the affected limb and also usually in the opposite limb, followed by ~15–30 MBq intradermal–subcutaneous Tc99m colloid. Both intradermal and subcutaneous injections are employed, and we usually use a combination of the two. Subfascial injections, for example, intramuscular injections, can be employed if the deep lymphatic system is to be evaluated. Injection is followed by mild exercise of the limbs and/or ambulation. Images are usually acquired in whole-body format at ~15–20 min, at ~2–3 hr, and sometimes at 6 to 24 hours after injection. The radiation dose to the patient is low from the small doses of Tc99m that are employed.

The choice of radiopharmaceutical can influence the rate of visualization of the draining lymph nodes. After interstitial injection, colloidal particles of appropriate size (in the range of ~5–200 nm) gain entrance to lymphatics through clefts in the walls of lymphatics (Fig. 1), or by

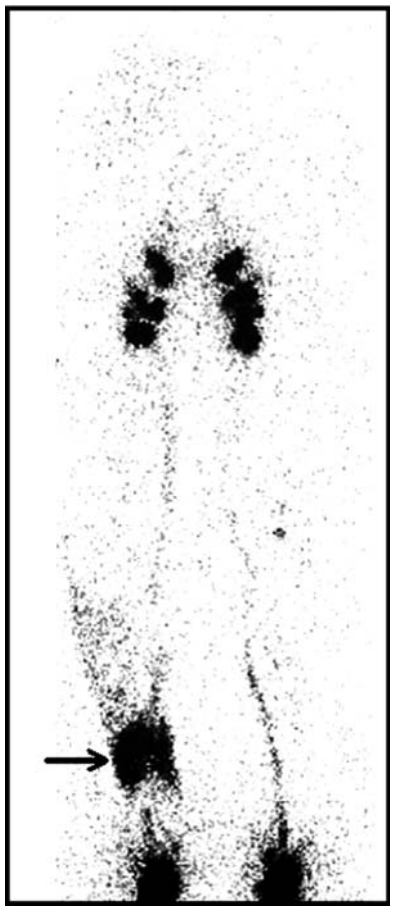

Fig. 17. Lymphoscintigram demonstrates regional retention of tracer in the right lower leg in area of cellulitis (*arrow*), despite presence of intact lymphatic vessels and nodal structures in both lower extremities.

endocytosis through the walls. Peripheral macrophages may phagocytose some particles. Intralymphatic particles may thus be either intracellular, phagocytosed by macrophages, or remain suspended in lymphatic fluid *(75)*. Either way, they are transported to the nearest draining lymph node, that is the sentinel node, of the tissue into which they were injected. In general, their rates of transport vary inversely with the size of the colloid particles, with faster transit observed for colloids with smaller particles *(76–79)*.

Once inside a lymph node, particles may be phagocytosed by macrophages or dendritic cells, which facilitates their intranodal retention and localization by imaging or by radiosensitive probes in surgery. In a typical injection used for sentinel node mapping, however, roughly 20–100 μg in the case of sulfur colloid, will be injected. If ~1% reaches the nearest draining node, however, roughly 0.2–1.0 μg or 10^{10} particles would be presented to the node for potential phagocytosis. This would likely overwhelm the phagocytic capacity of macrophages in a single lymph node. As a result, the observed retention of colloidal tracers within sentinel lymph nodes probably also represents retention and trapping by mechanisms other than phagocytosis, including nonspecific mechanical trapping in the reticular meshwork of the node. With time, however, such nonspecific trapping will allow release of particles for subsequent migration to other nodes *(76)*.

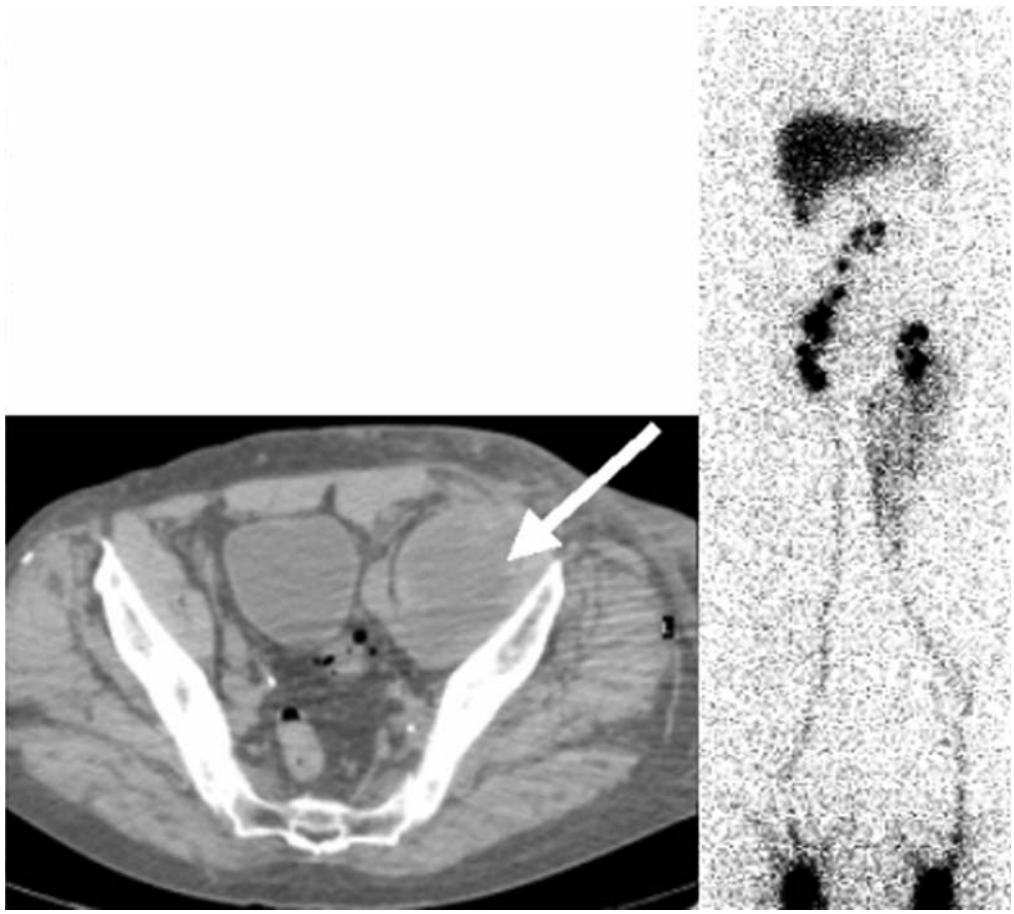

Fig. 18. Large synovial cyst (*arrow*) arising from left hip after arthroplasty resulted in stretching and compression of lymphatic and blood vessels and was accompanied by LE (demonstrated by lymphoscintigram in *right panel*) that responded to decompression of the cyst.

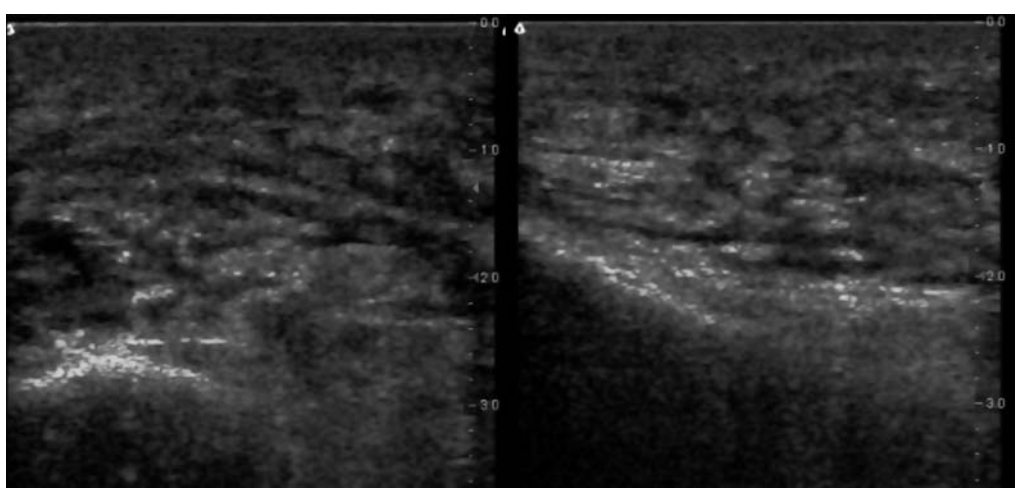

Fig. 19. Ultrasound images taken at level of ankle in an individual with LE. *Left*: Image before treatment with manual lymphatic drainage and external compression. *Right*: Image at same location after treatment, demonstrating reduced epifascial thickness.

Normal LS demonstrates prompt flow to inguinal or axillary nodes by 15–20 min in discrete, nontortuous lymphatics, and, in a minority of patients, to intercalated nodes (e.g. popliteal or epitrochlear nodes). Images at 2–3 hours demonstrate fairly intense inguinal or axillary uptake, with moderate uptake in liver and spleen (right panel, Fig. 4). Uptake in Virchow's node is a normal and common, but not invariant, finding. Abnormal findings include slow or no flow (Figs. 4, 5, and 11), tortuous or deviated channels, collateral or crossover flow, localized or diffuse dermal retention (Figs. 11, 17, and 18), and in the presence of chylous reflux, reflux into the contralateral extremity after unilateral injections *(74)*. Areas of inflammation or infection usually demonstrate increased retention of radiotracer, probably as a reflection of macrophage activation, increased numbers of phagocytic cells, and regional stasis and edema with delayed clearance of interstitial fluid (Fig. 17).

Applications of LS include evaluation of edema or limb enlargement, limb swelling with recurrent infections, assessing lymphatic deficiencies in primary LE, differentiating primary and secondary LE, determining the relative contributions of adiposity, lipedema, and LE in obese patients, demonstrating lymph reflux *(74)*, differentiating venous edema from LE (Fig. 6), and evaluating regional edema after liposuction or surgery in the limbs, axillary regions, inguinal regions, or abdomen/pelvis or head and neck. Diagnosis can be difficult and delayed in obese patients. Undiagnosed or untreated LE is associated with progressive disease, recurrent infections, and resistance to therapy. Patients incorrectly diagnosed with LE can receive inappropriate treatment. Congenital LE is variable in time of onset and severity, and increases surgical risk. For these and other considerations, an early and accurate diagnosis of the condition is important. LS is a valuable diagnostic tool in these and other similar clinical scenarios.

LS for planning therapy in patients with LE requires consideration of the pathophysiology in the particular patient as well as relevant technical factors such as the properties of the radiopharmaceutical employed, imaging times after injection, and patient activity after injection.

14. SUMMARY AND CONCLUSIONS

LE results from an imbalance in the generation versus removal of interstitial fluid and macromolecules. Acquired and congenital LE are highly variable in time of onset and severity of expression. LE often appears after provocative factors trigger an imbalance in interstitial fluid dynamics. It is important to establish the diagnosis of LE in contrast to other conditions that often mimic it, such as lipedema, phleboedema, or disuse edema. Edema is well demonstrated with MRI and CT, and can also be evaluated with ultrasound of the epifascial compartment, which is most commonly involved with LE. The severity of lymphatic functional deficits and mapping of lymph drainage can be readily evaluated with radionuclide LS. Lymphatic imaging plays a pivotal role in defining the etiology of extremity swelling and in predicting the success of conservative treatment. LE in cancer patients may result from obliteration or destruction of lymphatic tissue from surgery and/or radiation. When their use can be safely curtailed in the management of malignancies, the incidence and severity of LE can be reduced. In this regard, sentinel lymph node procedures can mitigate the impact of iatrogenic LE in populations and individuals where complete lymph node dissections can be replaced by sentinel node sampling alone. LE is a treatable disorder, and early recognition and treatment should be promulgated.

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14 3D-CT Lymphography for Mapping Metastatic Breast Sentinel Node and Axillary Nodes

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Contents

ABSTRACT

Background: The detailed relations between lymph nodes and lymph flow in the breast and the axilla can be clarified using 3D-CT lymphography (LG). We have developed this 3D image-processing system to depict more precise anatomical structure of mammary lymphovascular system. It enables us to perform systematic collection of axillary lymph nodes including sentinel nodes (SNs), and will decrease unnecessary lymph node dissection, even if the SNs have metastasized, and can decrease complications. We reviewed a validity of 3D-CT LG, and a metastatic pattern of axillary nodes and SNs of 40 metastasis-positive cases among 186 SN biopsies.

Methods: 3D-CT LG was performed on the day before the surgery to mark SN on the skin. Above the tumor and near the areola, 2 ml of Iopamiron 300 was injected subcutaneously. Images from a 16-channel multidetector-row helical scan were taken at 1 min after injection for SN detection, and at 3 and 5 min for observing advancement of lymph flow into venous angle. They were reconstructed to produce a 3D image of lymph ducts and lymph nodes by shaded volume-rendering method. The axillary lymph node groups from SNs to subclavicular nodes were exposed by partially removing fat tissue and muscle in the 3D image. SN biopsy was performed by dye-staining method using endoscopy. The SNs were found by following the dye in the lymph ducts on video monitor. SNs and the second and the third nodes were sampled by the same way.

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Results: We performed SN biopsy with 3D-CT LG in 146 patients. 3D-CT LG showed the precise lymphatic flow from the tumor to SNs. The position of SNs was identified by their surrounding pectoral muscles and vascular systems, such as axillary vein, lateral thoracic artery, and thoracodorsal artery. We classified the relationship between the lymph ducts and the drained SNs into four patterns: single duct to single node ($n = 88$); multiple ducts to single node ($n = 29$); single duct to multiple nodes ($n = 2$); and multiple ducts to multiple nodes ($n = 27$). 3D-CT LG can show sentinel lymph node at only 1 min after injection. But following up to 3 and 5 min after injection, we can follow the lymph ducts after the SLN into the second and third nodes toward the venous angle with the complex plexus in 3D-CT LG. The figure shows five bead-like-grouped lymph nodes beyond the SLN and lymph duct plexuses between them. Detection rate was 100% for SN; 80.1% for the third group; and 30.1% for the fifth group. Multiple nodes were observed in SN (19.9%), in the second group (10.1%), and in the third group (4.3%). SN metastasis was positive in 40 patients, and only SN metastasis was found in 21 patients (52.5%) among them; SN and second group metastases were in 19 patients; SN and second and third group metastases were in 16 patients; and beyond the third group metastasis was in 11 patients. But any skip metastasis beyond second and third nodes was not observed.

Conclusions: By 3D-CT LG, we can recognize the accurate and more precise lymph flow and their positional relations to surrounding anatomical architecture. It helps us easily to pursuit lymph flow and to remove sentinel, second, and third nodes by endoscopic technique. 52.5% of SN metastasis-positive patients had no metastasis in other nodes. They can be candidates for preserving their axillary node. 3D-CT LG-guided SN biopsy for the second and third groups will predict their existence and help us to avoid dissecting needless nodes.

Key Words: endoscopic surgery; sentinel node biopsy; axillary node mapping; lymphography; 3D-CT; breast cancer

1. INTRODUCTION

In early breast cancer, the presence of metastasis in axillary lymph nodes is an important factor in prognosis and further treatment. However, axillary lymph node dissection causes many complications, such as contracture of the shoulder joint, lymph edema, and paralysis of the upper extremities *(1)*. Convention holds that there is no need to dissect axillary lymph nodes for node-negative patients. To avoid unnecessary axillary lymph node dissection, sentinel node biopsy (SNB) has been performed *(2,3)*. SN is defined as the first lymph node drained of lymph flow from the tumor *(4,5)*. SNB can detect such metastases and provide information that may obviate the need for axillary lymph node dissection. The most commonly used methods to identify the SN are dye staining *(6,7)* and radioisotope incorporation *(8,9)*. Multidetector-row 3D-computed tomography (3D-CT) and mammary lymphography (LG) can be used to mark the precise location of the SN on the skin before the operation *(10–12)*.

The detailed relations between lymph nodes and lymph flow in the breast and the axilla can be clarified using 3D-CT LG *(13,14)*. We have developed this 3D image-processing system to depict more precise anatomical structure of mammary lymphovascular system. It enables us to perform systematic collection of axillary lymph nodes including SNs, and will decrease unnecessary lymph node dissection, even if the SNs have metastasized, and can decrease complications.

Previously, we devised an endoscopic surgical procedure for breast diseases; video-assisted breast surgery (VABS) *(15)*. VABS is a less invasive and esthetically a better operation for benign and malignant breast diseases. In this study, we assessed the validity of 3D-CT LG in SNB

of 186 patients, investigated the extent of metastasis style in 40 patients who were metastasis-positive based on the novel technique, and applied the technique to SNB using the dye-staining method and 3D-CT LG guidance.

1.1. Patients

Since July 2002, SNB was performed in 186 patients, with SNB using VABS and 3D-CT LG being performed in 146 of these. Patient characteristics are shown in Table 1.

1.2. 3D-CT LG

Interstitial 3D-CT LG was performed using a 16-channel multidetector-row helical 3D-CT scanner (Toshiba Aquilion 16; Toshiba Medical Systems Corporation, Tochigi, Japan). Patients were placed in the supine position with arms positioned in the lateral abduction direction, suitable for the operating position. After local anesthesia by subcutaneous injection of 0.5 ml of 1% lidocaine, 2 ml of iopamidol (Iopamiron 300; Nihon Shering, Osaka, Japan) was injected intracutaneously into the periareolar skin and the skin above the tumor. At 1 and 3 min after injection (sometimes 5 min for observing advancement of lymph flow), a CT image was taken with a 3-mm slice thickness. SNs were identified on transaxial CT images, and their location was marked on the skin surface with an oil-painting pen using a laser pointer of CT on the day before the surgery. 3D-CT images were then reconstructed from transaxial enhanced CT images, which clearly showed the lymph ducts and SNs.

1.3. Surgical Methods

VABS has been described in detail previously *(15)*. The operative procedures were as follows: skin incision in the axilla and/or periareolar, skin flap formation via the tunnel method *(16)*, pectoral muscle fascia dissection, vertical section of the mammary gland,

Table 1
Patient Characteristics[a]

	Mean	*Range*
Age (y/o)	52.7	26–85
Tumor size (cm)	2.2	0.1–10
	Number	%
Tis	3	1.6
T1a/T1b/T1c	3/20/86	1.6/10.8/46.2
T2/T3/T4	53/9/12	28.5/4.8/6.5
Lymph node metastasis (N)	40	21.5
Distant metastasis (M)	9	4.8
ER (±)	122/64	65.6
PgR (±)	98/88	52.7
HER2 (±)[b]	44/142	23.7
Total	186	

ER, estrogen receptor; PgR, progesterone receptor; HER, human epidermal growth factor receptor

[a] Modified from *(14)*.

[b] HER2: human epidermal growth factor receptor type 2; HER2+ means Herceptest 3+ and 2+; HER2– means Herceptest 1+ and 0.

SNB by the dye-staining method guided by preoperative 3D-CT LG marking, and axillary lymph node dissection (levels I and II). Radiotherapy and chemotherapy were performed for malignant diseases.

SLNB was performed by the dye-staining method using a part of VABS technique at the beginning of the operation, before gland resection. In the periareolar region and over the tumor, 2 ml of 1% indocyanine green was injected subcutaneously. A 1-cm long skin incision was made along wrinkles in the axilla at the position marked by 3D-CT LG. A Visiport optical trocar (Tyco Healthcare Japan, Tokyo, Japan) was inserted into the incision after 20 min. The endoscopic view was observed through Visiport with a 10-mm diameter, straight-angled rigid endoscope (Olympus Optical, Tokyo, Japan), and the stained lymph nodes were found by following the dye in the lymph ducts. The lymph nodes were sampled and metastasis was determined on fast-frozen sections. Axillary lymph node dissection was performed at levels I and II with bipolar scissors through the same incision that was lengthened to 2.5 cm. The inferior pectoral nerve, long thoracic nerve, second and third intercostobrachial nerves, thoracodorsal nerve, artery, and vein were observed and preserved. The lateral pectoral artery was preserved for the lateral tissue flap. After surgery, SNs and axillary lymph nodes were pathologically examined by standard H&E staining.

Informed consent to the procedure was obtained from all the patients before surgery.

2. LYMPH FLOW FROM TUMOR TO SN *(13)*

The lymph flow of the whole breast has been reported to collect into a subareolar plexus and then drain toward the axilla via lymph-collecting ducts, by human cadaver studies *(17,18)*. It became the theoretical basis for the subareolar injection of dye and/or isotope for lymphatic mapping as part of the SN biopsy for breast cancer *(19,20)*. On the other hand, the individual lymphatic flow is not identical in each living patient studies of SN biopsy.

3D-CT LG showed the precise lymphatic flow from the tumor to the SN (Fig. 1). In Fig. 1a, the lymphatic flow from the tumor was divided into the periareolar and directly to the axilla. The periareolar lymphatic flow drained from the tumor and circled around the nipple and went

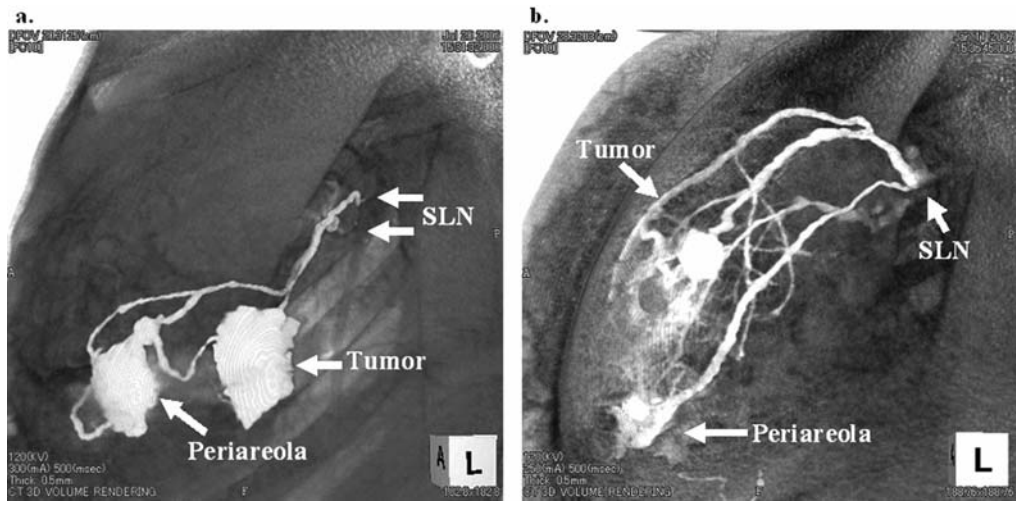

Fig. 1. Visualization of SNs and lymph ducts (LD) using 3D-CT LG *(13)*. Iopamidol was injected intracutaneously into the periareolar skin and the skin above the tumor. (**a**) Two LDs draining into two SNs. (**b**) Multiple LDs draining into a single SN.

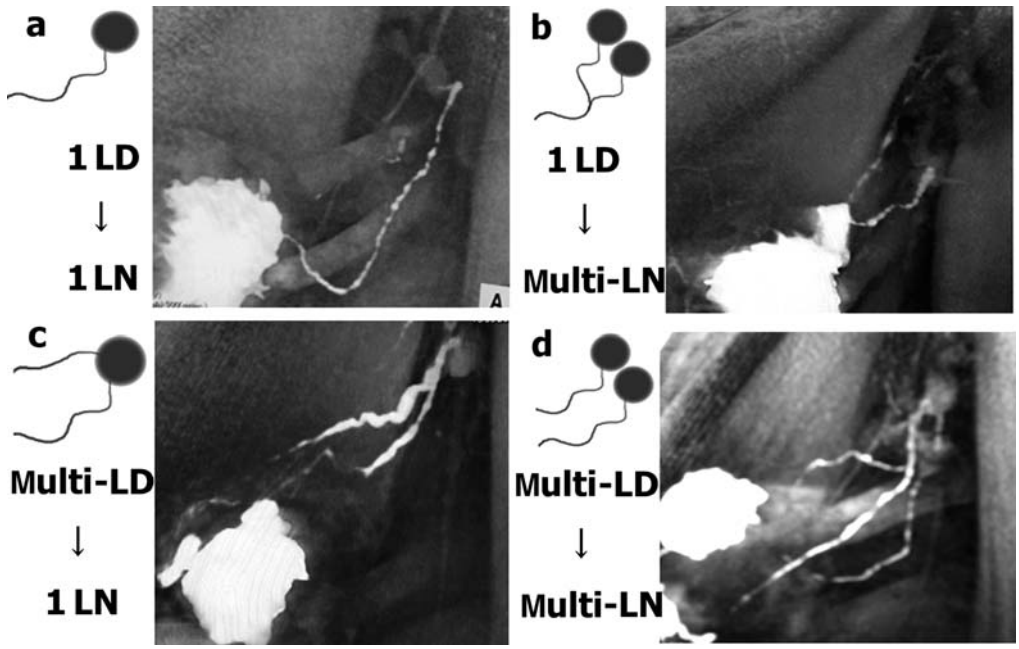

Fig. 2. Four patterns of relationship between the lymph duct and the sentinel lymph node *(14)*. The lymph flow pattern can be classified into these four types.

to the axilla. This lymph duct to the axilla was separated from the direct duct from the tumor. In Fig. 1b, the lymphatic flow was multiple and complicated, but the basic structure was same as that of Fig. 1a.

We classified the relationship between the lymph ducts and the drained SNs into four patterns *(13)* (Fig. 2), according to the classification by Suga et al. *(21)*. A single duct to single node pattern was observed in 88 cases (60.2%), multiple ducts to a single node in 29 cases (19.9%), single duct to multiple nodes in two cases (1.4%), and multiple ducts to multiple nodes in 27 cases (18.5%).

The internal mammary SN was also detected in five patients, but the rate was lower than that of the peritumoral injection *(22)*. We are trying to improve it by the injection of the contrast medium iopamidole into the retromammary space behind the tumor. The peritumoral injection, which is recommended in radioisotope technique, is not suitable for 3D-CT LG, because the contrast medium is needed to use much volume as 1 ml and it may flow into surrounding mammary duct systems.

SN is typically detected using dye staining or radioisotope incorporation. However, the tract from the tumor to the SN cannot be observed clearly by either methods *(23,24)*. We cannot detect whether dye-stained or hot-spotted nodes are really the first lymph nodes. This may be the basis for false negatives in SN biopsy. Lymphoscintigraphy may only show the main negative lymph node, and cannot clearly visualize the direct connection of SN and their afferent lymph ducts, because of slow lymphatic migration of radiocolloids, and because of the limitation of spatial resolutions and the lack of anatomic landmarks. In contrast, 3D-CT LG can demonstrate the precise route of the lymph duct and the exact location of the SN with the detailed surrounding anatomic structures, and since it does not involve the use of radioisotopes, it can be done at any institution that is equipped with a CT scanner. Therefore, 3D-CT LG is a very useful examination, which is essential for SNB.

Interstitial injection of iopamidol had no adverse effects locally and generally. The pathological metastatic status of SNs and axillary lymph nodes was as follows. No SN was identified in five patients of the 40 SNBs using only the dye-staining method, and the detection rate was 87.5%. However, all SNs were identified in 146 SNBs using the dye-staining method with 3D-CT

LG marking. This detection rate was 100%. Backup axillary dissection was performed in each of the 40 SNB patients in the early phase according to both methods of SNB. One false-negative case occurred only in the dye-staining method (12.5%), but no false-negative case was found in 3D-CT LG (0%). The average sampled number of SNs was 1.7 in the cases without 3D-CT LG and 2.3 in the cases with 3D-CT LG.

Four patterns of lymph ducts and SNs have been revealed using 3D-CT LG *(13)*. The lymph ducts to the SN are complicated. For example, in this study, we observed that in over 60% of cases, many ducts joined together into a single duct to form a single SN. However, more than two SNs were shown in 19.9% of the cases. These may have been missed without 3D-CT LG guidance. Figure 4a shows a typical example of a multinode pattern, in which three different lymph nodes were all SN, each from a different lymph duct. The node from the main thick duct was not metastasized; however, the other two from the narrow collateral duct were metastasized. Thus, the sampling of all three nodes is necessary. The dye-staining method and the isotope method of SNB could not reach as such the latter collateral nodes. These might become false-negative study. Therefore, 3D-CT LG is effective in raising accuracy of SNB.

3. LYMPH FLOW FROM SN TO AXILLARY ANGLE IN AXILLA *(14)*

The procedure of 3D-CT LG takes only about 1 min after injection of iopamidol subcutaneously over the tumor and around the nipple for representing SN precisely. Examination of iopamidol flow 1, 3, and 5 min after injection revealed that the flow extended over the SN into the next nodes, and into the venous angle in half of the patients examined. Therefore, we can easily ascertain the tracts that cancer cells will spread through during metastasis. We defined these tracts as the second and third SNs. As a representative example, (Fig. 3 and Color Plate 8)

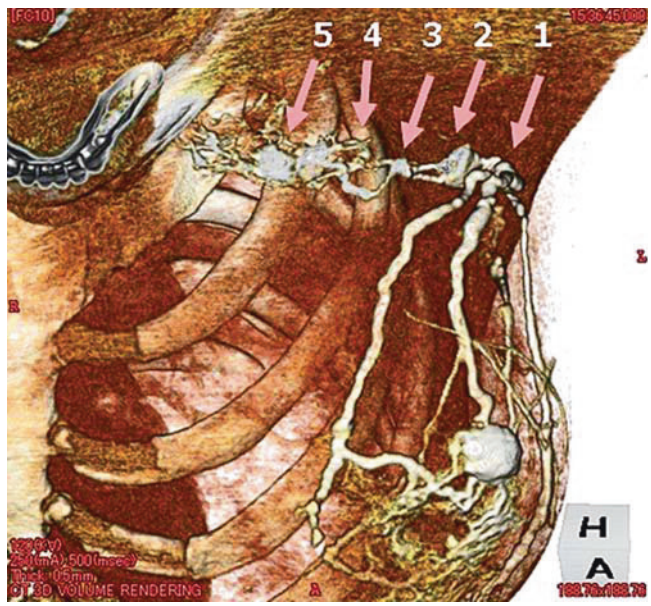

Fig. 3. Chronological examination of 3D-CT LG *(14)*. 3D-CT LG was examined 1, 3, and 5 min after iopamidol injection. Iopamidol flowed to extend over the SN into the next nodes. Five bead-like grouped lymph nodes in the axilla can be visualized by partially removing the pectoral muscle in the CT monitor. These are thought to be the order of lymph metastasis. *Arrows* point to lymph nodes 1–5 after SN. (*see* Color Plate 8)

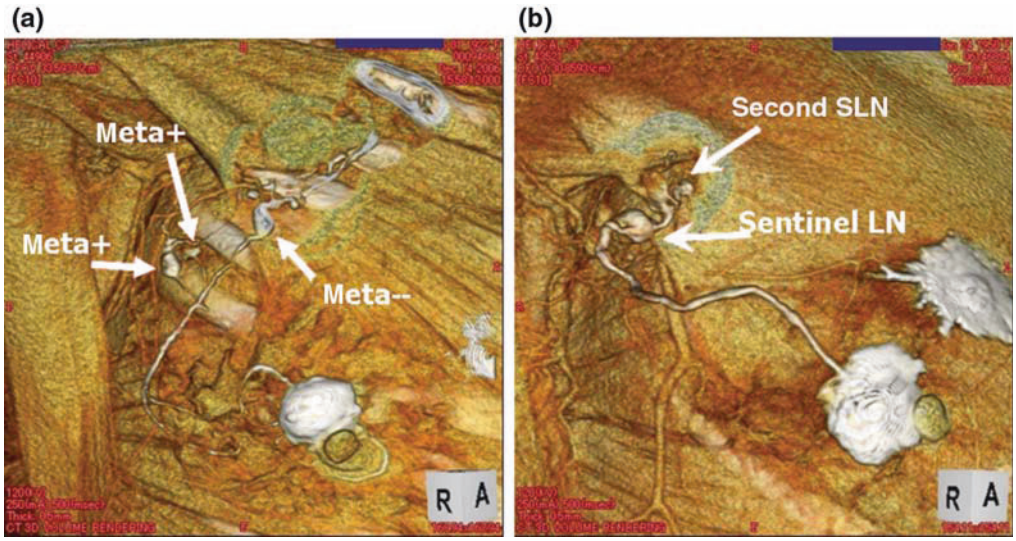

Fig. 4. Detection of SN metastasis *(14)*. (**a**) Three SNs are recognized by 3D-CT LG. The right node drained from the main lymph duct was not metastasized. On the other hand, the other two nodes drained from the narrow collateral duct were metastasized. Dye and isotope could not reach as such the latter collateral nodes. These might become false-negative study. Therefore, 3D-CT LG is effective in raising accuracy of SNB. (**b**) The SN was metastasized, but the second node and the other nodes were all negative. (*see* Color Plate 9)

shows a clear view of five bead-like grouped lymph nodes beyond the SN into the center of the body, and lymph duct plexuses between them. They are thought to imply the order of lymphatic route of metastasis. (Figure 4a and Color Plate 9) shows three separated SNs, which were drained from three different lymph ducts diverging from one duct. Two of three separated SNs were positive for metastasis, but the other SNs from the main lymph duct were negative. Figure 4b shows two chained SNs. The first SN was positive for metastasis, but the second was negative.

Since December 2001, we performed VABS in 230 patients, SN biopsy in 186 patients, and 3D-CT LG in 140 patients. Table 2 shows the pathological status of metastases in SNs and axillary lymph nodes. SN metastasis was positive in 40 patients; of these, 21 patients experienced metastases solely in the SN. Except for SN, only the second axillary lymph node group was metastasized in 3 patients, the second and the third node groups were metastasized in 5 patients, and more than three groups were metastasized in 11 patients. It was confirmed that these metastases occurred in order of lymph flow presented by the lymphoid path of these 3D-CT LG (Figs. 3 and 4).

The second and third lymph node groups could be recognized by 3D-CT LG and selectively removed by VABS. Table 3 shows the pathological status of their metastases and the other axillary lymph nodes in 40 SN metastasized patients. If the second and third lymph node groups' biopsy was performed, it was prognostic for other axillary lymph node metastasis. Its accuracy, sensitivity, and false-negative rate were 100, 100, and 0%, respectively.

Even if the patients presently examined had metastasis of SN, about half of them had no metastasis in axillary lymph nodes (21 among 40 patients, 52.5% in Table 2). In these patients presenting with only SN metastasis, sufficient information can be obtained regarding the lymph node status of cancer staging to plan primary therapy after surgery, avoiding axillary lymph node dissection. Since the absence of other metastases is crucial to this approach, we need to

Table 2
Metastatic Status of Sentinel and the Other Axillary Nodes[a]

		No. of axillary LN metastasis[b]			
Sentinel LN metastasis		*2nd*	*3rd*	*4th<*	*Sum*
Positive	40	19	16	11	19
Negative	141				
Not detected	5	0	0	0	0
Total	186	19	16	11	19

Detection rate 97.3% (without 3D-CT 88%/40, with 3D-CT 100%/146).
Average LN number 2.0, Only SLN metastasis 21 (52.5%).
LN: lymph node, 3D-CT: 3D-computed tomography, LG: lymphography.
[a]Modified from *(14)*.
[b]Axillary LN metastasis: SLN is not included.

Table 3
Metastatic Relations of second and third SNs to the Other Axillary Nodes[a]

Status of second and third SLN metastasis		*Status of axillary LN metastasis[b]*	
		Negative	*Positive*
Positive	19	8	11
Negative	21	21	0
Total	40	29	11

LN: lymph node
Second and third SLN biopsy: Accuracy = 100%, False-negative rate = 0%, Sensitivity = 100%
[a]Modified of *(14)*.
[b]Number of Axillary LN metastasis: Primary, second, and third SLNs are not included.

analyze how it would be possible to conclude that SLN metastasis is unique. We suggest that this can be confirmed by collecting lymph nodes systemically based on the 3D-CT LG-acquired map of lymph nodes and ducts beyond SN. Histological examination of fast-frozen sections of the second and third lymph nodes during the operation will provide the information to omit axillary lymph node dissection.

4. METASTATIC EVALUATION BY 3D-CT LG

3D-CT LG may predict whether SN is metastasizing or not. When occupied with cancer cells, 3D-CT LG shows only the trumpet-like inflow portion of the lymph node, but the node is still recognizable. Sometimes, the lymph duct detours around the metastasized lymph node. However, in partial metastasis of the lymph node, 3D-CT LG shows no apparent difference from normal nodes. We have to examine the pattern of the duct route and the enhanced pattern of lymph node more carefully to predict metastasis. Ultrasonography and magnetic resonance imaging show only morphology and blood flow, and cannot reveal metastasis. While positron emission tomography can detect some metastases, those that are small escape detection. Of all these techniques, 3D-CT LG is superior.

5. ENDOSCOPIC SNB GUIDED BY 3D-CT LG

We have performed endoscopic surgery, named as VABS, for all breast surgical procedures through a small wound port in the inconspicuous axillary area or periareolar *(15,25)*. VABS is also used for SNB. The incisional wound is only 1 cm long and inconspicuous, without any complications. However, precise information is needed about the location of SN, because the method relies on endoscopic vision. In five cases, we could not find the SLN using only the dye-staining method. 3D-CT LG guidance helps in finding the SN easily by giving precise information. The structure of the dye-stained lymph ducts and SN was exactly the same on the endoscopic view as with the 3D-CT LG.

The lymph ducts to the SN are complicated. In most cases, many ducts join together into a single duct to a single SN (60.6%). However, more than two SNs were shown in 19.7%, and these may have been missed without 3D-CT LG guidance, and hence 3D-CT LG is indispensable for SNB.

6. CONCLUSION

By 3D-CT LG, we can recognize the accurate and more precise lymph flow in the breast and the axilla. Even in patients with SN metastasis, if we find no metastatic presence in the second and third SNs, the need to dissect more nodes is obviated. In the near future, it will be necessary to omit axillary dissection in such patients.

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15 Molecular Imaging of Neuroendocrine Cancer by Fusion SPET/CT

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CONTENTS

ABSTRACT

Neuroendocrine (NE) cancers are usually suspected on clinical symptoms related to their metabolically active peptide secretion into the circulatory system. The most effective treatment is surgery and a preoperative accurate localization of these slow growing tumors is needed. Combined anatomical (CT) and molecular imaging modalities using single-photon emission computed tomography (SPET) with radiolabeled pentetreotide have been developed in routine, and we report here the potential of SPECT/CT image fusion for diagnosis, staging, and evaluation of treatment efficacy of NE cancers.

Patients: Sixty-four consecutive patients were included: 2 patients with MEN 2b, thoracic lesions ($n = 5$), bowel lesions ($n = 28$), pancreatic lesions ($n = 17$), and unknown primary endocrine tumors ($n = 12$). Age ranged from 31 to 64 years.

Imaging Protocol: Hybrid images of functional SPET and anatomical CT data were acquired using a SPET/CT system combining a dual-detector gamma camera with a low-dose CT (Millennium VG & Hawkeye GE Healthcare), 24 hours after intravenous injection of 200 MBq ^{111}In-pentetreotide and compared with additional whole-body and planar conventional scintigraphies obtained, respectively, at 4 and 24 hours. All images were reviewed without knowledge of clinical data and the results correlated with the follow-up.

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Results: Thirty-four were true positives, twenty-one true negatives, and nine remained equivocal. Eighty-eight uptake sites in the neck, thorax, liver, abdomen, and skeleton were detected. Sensitivity and specificity raised, respectively, from 81 and 22 without fusion to 92 and 80% with fusion ($p = 0.005$). Equivocal foci decreased from 17% without CT to 5.7% with CT. Site-by-site interobserver performances were also improved from 60 to 88% when using CT fusion ($p < 0.005$).

Conclusion: Molecular imaging of NE cancer by fusion SPECT/CT is an accurate modality to precise tumor site location and has a significant impact in the management of endocrine tumor patients.

Key Words: neuroendocrine tumor; molecular fusion imaging

1. INTRODUCTION

Neuroendocrine (NE) cancers constitute a heterogeneous group from well-differentiated to undifferentiated solid tumors arising from NE cells. These tumors are often localized in the abdomen and usually suspected on clinical syndromes (functioning tumors) related to their metabolically active peptide secretion into the circulatory system or may only exhibit elevated serum markers *(5,19)* with no specific clinical syndrome (nonfunctioning tumor). They have generally a limited growing rate. The curative or debulking surgical approach is the most effective treatment, while medical treatment with somatostatin analogues and interferon is used when metastases have occurred *(16)*. Therefore, a preoperative accurate localization is needed and requires to perform additional investigations. Anatomical imaging as computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound are mainly suited to detect lesions when morphologic alterations occur *(15,18,21)*. However, these methods failed to correctly identify abdominal tumors from intestinal structures *(7,8,12)*. Functional imaging modalities and specifically single-photon emission computed tomography (SPET) with radiolabeled pentetreotide which binds to somatostatin receptors (SRS SPET) have been developed in routine (or are routinely used) and may detect lesions before anatomical imaging methods *(6,11,13)*. Anatomical and functional imaging modalities provide complementary information and the interpretation of functional images may, therefore, benefit from coregistration with anatomical views. The combination of sequential SPET using a hybrid system of gamma camera and CT in a single exam facilitates the anatomic localization of the tracer uptake *(10)*. We report here the role of SPET/CT image fusion using somatostatin receptor scintigraphy (SRS SPET/CT) for diagnosis and staging, in 64 patients with NE cancers.

2. MATERIAL AND METHOD

2.1. Patients

Sixty-four patients with histologically proven endocrine tumors were included: and consisted in 2 patients with MEN 2b, thoracic lesions ($n = 5$), bowel lesions ($n = 28$), pancreatic lesions ($n = 17$), and unknown primary endocrine tumors ($n = 12$). Age ranged from 31 to 64 years (51.2 ± 14.5 years). At diagnosis, all patients underwent ^{111}In-pentetreotide SPET/CT (SRS SPET/CT) additionally to conventional imaging at initial workup.

2.2. Imaging Protocol

Hybrid images of functional SPET and anatomical CT data were obtained using an SPET/CT system combining a dual-detector gamma camera with a low-dose CT

(Millennium VG & Hawkeye GE Healthcare). SPET images (360°) were acquired 24 hours after the intravenous injection of 200 MBq of ^{111}In-pentetreotide on a 64 × 64 matrix (two energy peaks of Indium, 6° angle step, 40 s per frame, and 128 planes of 4 mm each) with medium-energy collimators. Additional whole-body and planar conventional scintigraphies were respectively obtained at 4 and 24 hours. Reconstruction was performed by filtered back projection (FBP) or iteratively using the ordered subsets expectation maximization (i.e., OSEM) technique with attenuation correction (IRAC) or without attenuation correction (IRNC).

Fusion of both anatomical and functional images was finally obtained in transaxial, sagittal, and coronal planes using the workstation software (eNTEGRA and GE Healthcare) with and without X-ray attenuation correction.

SRS SPET/CT was performed in the initial workup, in combination with conventional imaging including ultrasound, CT, MRI, and selective arteriography. Each patient had a minimal follow-up of 12 months.

A correlation with clinical data, conventional imaging results, and follow-up was performed in order to classify SRS uptake sites as true or false positive, true or false negative, or equivocal.

2.3. Data Analysis

All images were reviewed by two independent observers without knowledge of clinical data and results of whole-body and planar SRS images.

A first analysis consisted on the comparison of image quality of SPET images obtained with the three different reconstruction modalities: FBP, iteration with attenuation correction (IRAC), and without attenuation correction (IRNC).

Secondarily, we analyzed the additional information provided by SRS SPET/CT as compared with conventional imaging.

Then, we compared SPET/CT images alone (without interpretation of combined CT images) to SPET images in each patient.

2.4. Statistical Analysis

The χ^2-test was used to compare the interobserver accuracy. A two-tailed p-value < 0.05 was considered statistically significant. We did statistical analysis by using the SPSS package version 12.0. Interobserver accuracy was calculated as well as the clinical relevance of fusion imaging as compared with other imaging results and with the follow-up.

3. RESULTS

3.1. Image Quality

The best quality was obtained with iterative reconstruction with attenuation correction (IRAC). In many (41%) cases, this reconstruction method demonstrated small abnormal findings not detected when using the other two reconstruction methods (Fig. 1 and Color Plate 10).

There were 34 true-positive and 21 true-negative scans and SRS SPET/CT remains equivocal in nine patients. Eighty-eight uptake sites were detected and localized in the neck (5), chest (8), liver (28), abdomen (39), and skeleton (8). As compared to conventional imaging, SRS SPET/CT founded twice more distant lesions in the abdomen.

Sensitivity and specificity were calculated for each observer on SPET images (without knowledge of combined CT images) and on SPET/CT fusion images. Finally, equivocal cases were classified as false-positive cases. Results are shown in the Table 1.

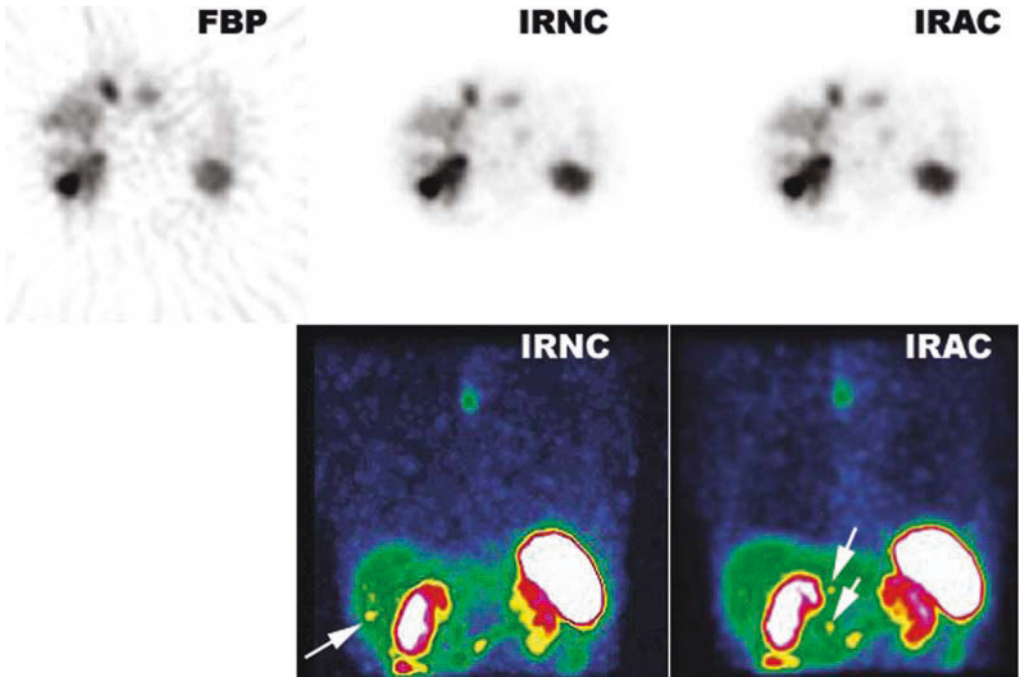

Fig. 1. Image quality analysis using three different reconstruction softwares: filtering back projection (FBP), iterative reconstruction without attenuation correction (IRNC), and iterative reconstruction with attenuation correction (IRAC). *White arrows*: liver metastases nondetected by FBP reconstruction. (*see* Color Plate 10)

3.2. Equivocal Exams

Using SPET/CT fusion images, the number of equivocal foci decreased from 17 to 5.7%, respectively, without and with combined CT interpretation. Site-by-site interobserver performances were also improved from 60 to 88% when using CT fusion ($p < 0.005$, χ^2- test).

Table 1
Comparison of Performance of SPET Alone and SPET/CT. Results are Those Obtained after the Analysis of 88 Abnormal Uptake Sites by the 2 Independent Observers

	SPET	*SPET/CT*
True positive	46 [52.3%]	58 [65.9%]
True negative	7 [7.9%]	20 [22.7%]
False positive	9 [10.2%]	0 [0%]
False negative	11[12.5%]	5 [5.7%]
Equivocal	15 [17.1%]	5 [5.7%]
Sensitivity	80.7	92.1
Specificity	22.6	80.0
Predictive positive value	65.7	92.1
Predictive negative value	38.8	80.0
Accuracy	60.2	88.6

3.3. Comparison Between SPET and SPET/CT

The classification as true-positive or true-negative cases was dramatically enhanced and sensitivity and specificity were significantly improved increasing from 81 and 22% for SPET alone to 92 and 80% for SPET/CT, respectively ($p < 0.05$, χ^2-test).

Nine uptake sites classified as false positive with SPET alone were in relation to digestive physiological uptake and classified as true negative with SPET/CT. Among 11 sites classified as false negative with SPET alone, 6 were associated with a mild pentetreotide uptake (reported to physiological elimination), while SPET/CT founded three abnormal uptake sites, localized in the liver ($n = 2$) and bone ($n = 1$). In 15 cases equivocal with SPET images alone, SPET/CT was positive in six of them and negative in nine others.

4. DISCUSSION

The treatment of NE tumors is essentially based on surgical removal of the primary tumor and isolated metastases. At initial staging, early and accurate detection of tumor sites are of great importance. However, the diagnosis of the disease is often made too late when the metastatic spread is important. Conventional imaging modalities used in diagnosis and staging of NE tumors include ultrasonography, CT, MRI, and selective arteriography *(15,18,21)*. Functional SPET based on receptor expression of the tumor (SRS SPET) is superior to these anatomical methods for identifying and assessing NE tumor *(6,13)*. In our study, the number of equivocal exams decreased from 17 to 5.7%, respectively, without and with interpretation of combined CT images. Site-by-site performances were also improved from 60 to 88% when using combined CT images. Similarly, with interpretation of combined CT images, the classification of true-positive or true-negative cases dramatically enhanced.

Some attempts using positron emission tomography (PET) with fluorodeoxyglucose have given poor results concluding that SRS remains the modality of choice for evaluating patients with NE tumors *(1,3)*. New tracers as C11-hydroxytryptophan, Cl1-/F18-trihydroxyphenylalanine, or F18-L-fluorodopamine (18F-FDOPA) have been developed and tested *(2,9,17)*. It is a very promising way as 18F-FDOPA PET seems to perform better than SRS in visualizing carcinoid tumors and may even do better than CT for bone lesions. However, SRS remains useful in noncarcinoid tumors and superior to 18F-FDOPA PET for the detection of liver metastases *(17)*. This point emphasizes the importance of a precise histological tumor characterization to optimize the imaging strategy.

In both SPET and PET studies, the lack of structural delineation and the lack of specificity are improved by hybrid imaging using SPET/CT and PET/CT. Despite the high sensitivity of SRS, the technique may be unable to precisely localize abdominal tumors. In order to obtain better localization of abnormal uptake, a correlation with high-resolution anatomic imaging modalities was suggested *(4,14)* and sophisticated softwares were developed to integrate nuclear medicine and CT or MRI studies. Using external or internal landmarks fusion images were obtained opening a new era for hybrid detection *(20,22)*. In the abdomen, SPET/CT imaging has demonstrated its ability to separate physiologic intraluminal bowel activity and abnormal tissue activity. Moreover with these methods, tumor sites localized in bone and bone marrow are easily detected.

In our experience, fusion of functional and anatomic images provides an additional value to SRS scintigraphy in the diagnostic and the staging of NE tumors, increasing sensitivity, specificity, and decreasing the rate of equivocal exams.

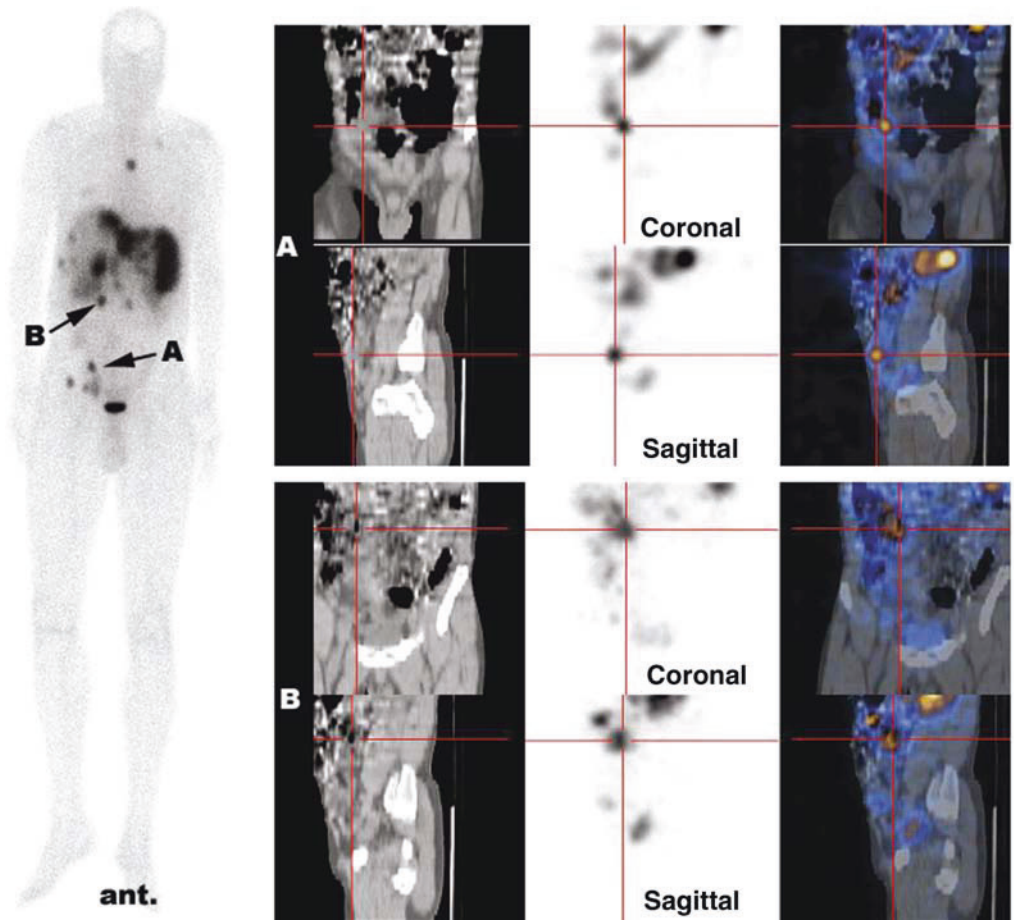

Fig. 2. SRS whole-body scan (*left*) in a patient with metastatic thymic neuroendocrine tumor demonstrated several foci of increased ^{111}In-pentetreotide uptake in the chest, and right upper and lower abdomen. Selected coronal and sagittal SPET/CT slices showed the precise localization of one of the areas of abnormal uptake in the right lower abdomen (**A**) and in the right upper abdomen (**B**). Fused images (*right*) enabled metastatic lesions. (*see* Color Plate 11)

5. CONCLUSION

Molecular imaging of NE cancer by fused SPET/CT and functional tracers (SRS SPET/CT) is an accurate modality to precise tumor site localization. SPET/CT improves interpretation quality and accuracy by lowering false-positive results, by increasing interobserver concordance, and must be systemically used for the upper abdomen.

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16 Functional Molecular Imaging of Prostate Cancer Lymph Node Metastases: Adenovirus-Mediated Lymph Node Detection

Saul J. Priceman, MS, Jeremy B. Burton, PhD and Lily Wu, MD, PhD

CONTENTS

ABSTRACT

Like other solid epithelial tumors, nodal involvement in prostate cancer signifies a poor clinical outcome. Yet, the lack of standard pelvic lymph node (LN)-sampling procedures and specific noninvasive imaging approaches to accurately assess nodal involvement is hampering the management decision. This chapter will discuss the debates in the clinical community on LN dissection and the development of various imaging modalities for prostate cancer. We will also describe a novel approach of using recombinant human adenoviral vectors (Ads) to directly map nodal metastases in experimental models of human prostate cancer. By exploiting the innate lymphotrophic properties of adenovirus and the prostate-restricted expression of imaging reporter genes incorporated into the viral vector, we were able to produce bioluminescent or positron emission tomography (PET) signals that correlate with the presence of metastatic lesions in the draining LNs. Significantly, this approach enables the direct PET visualization of sentinel lymph node (SLN) metastases, without the need for lymphadenectomy. We believe that developing and implementing more effective diagnostic imaging modalities to assess nodal status could improve the outcomes for patients with advanced stage prostate cancer.

Key Words: adenovirus; lymph node; metastasis; PET; luciferase; gene therapy

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1. INTRODUCTION

Among the clinical practitioners who treat prostate cancer, there is general consensus about the importance of lymph node (LN) status affecting patient outcome. However, unlike breast cancer and melanoma, there is no uniform practice of LN sampling to ascertain nodal status of prostate cancer. The possible reasons for the difficulties encountered in the workup plan for this disease might be several folds. The deep pelvic location of the prostate gland and its complex lymphatic drainage pattern presents clear hurdles to define the precise location and extent of LNs that need to be sampled. Clearly, a specific, noninvasive imaging technology that could accurately detect nodal involvement or guide the nodal assessment procedure for prostate cancer will be of great value. In the following sections, we will discuss the current status of lymphadenectomy in prostate cancer, overview the conventional noninvasive imaging modalities used to assess nodal status, and describe a novel viral vector-mediated prostate-specific imaging method to detect nodal metastases directly.

2. DEBATES ON LYMPH NODE SAMPLING IN PROSTATE CANCER

In patients with prostate cancer, clinical variables including serum prostate-specific antigen (PSA) levels, Gleason tumor grade, and digital rectal examination are useful in predicting outcome. Patients with Stage T2 or less, PSA < 20 ng/ml, and a Gleason sum of 6 or less have less than 10% likelihood of having nodal metastases. Among the adverse pathologic features of prostate cancer, the presence of pelvic LN metastasis is the strongest predictor of disease recurrence and progression *(1–4)*. For instance, the 10-year progression-free survival probabilities were 79% for organ-confined disease, which decreased to only 12% for disease with LN metastases *(1)*. At the present time, accurate LN staging can only be determined by pelvic lymphadenectomy *(5)*. Despite the importance of LN status on clinical management of prostate cancer, there are ongoing debates in the urologic oncology community on the value and best method to assess pelvic LNs, as a quick search of the US National Library of Medicine PubMed database will reveal over 15 editorial comments between 2006 and 2008 alone *(6,7)*. On the one hand, there is a growing sentiment that suggests extended pelvic lymphadenectomy increases the detection of positive LNs and survival *(3,8–10)*. Yet, on the other hand, several reports suggest that LN dissection does not affect disease outcome *(11,12)*. What might be the reasons for this discrepancy?

In other genitourinary malignancies such as penile, testicular, and bladder cancers, the clinical benefit of extended LN sampling and lymphadenectomy is well supported. Testicular cancer is a highly curable disease and surgical resection of the primary tumor and therapeutic retroperitoneal lymphadenectomy for both early- and advanced-stage diseases are integral components of management to achieving a successful outcome *(13)*. Results from retrospective analyses of a bladder cancer patient database *(14)* and a sizable clinical series *(15)* suggested that extended pelvic lymphadenectomy at the time of cystectomy not only provides the most accurate staging but also offers the patient the best chance of survival. The concept that sentinel lymph nodes (SLNs) are the first landing sites of cancer cells carried by lymph flow from a tumor was first proposed by Cabanas *(16)* in 1977 for penile cancer. In this disease, survival is clearly dependent on the LN status, rather than the stage of the primary tumor *(17,18)*. Moreover, therapeutic benefit of early inguinal lymphadenectomy is suggested by 5-year survival rates of up to 86% *(18)* as compared 8–24% in patients with late presentation of unresected nodal metastases *(19)*. The genuine importance of LN status in penile cancer has led to refined methods of nodal sampling by preoperative lymphoscintigraphy and dynamic sentinel node biopsy *(20)*. Why is the scenario different for prostate cancer?

Several clinically relevant issues are at the crux of this debate regarding the need and extent of LN dissection for prostate cancer. The points in support of performing more comprehensive pelvic LN sampling include the following: first, it is well supported and logical to assume that a more extensive pelvic LN dissection (PLND) will improve the yield of positive nodes and accuracy of staging, which in turn allows for better identification of patients who may benefit from more aggressive therapy *(9,10)*. A second important point is that excision of low-volume or limited LN metastases may transform the procedure into a potentially curative one, as suggested by several reports *(3,8,9)*. The major uncertainty about performing extended PLND at the time of radical prostatectomy is the benefit versus risk consideration of this procedure *(7)*. In a contemporary patient population in the post-PSA era, the incidence of nodal involvement is shifted down to below 10% in the prostatectomy patients *(21)*. Hence, a large proportion of patients who underwent the removal of draining LNs might not have benefited from this procedure. Besides, the increased cost and time of extensive pelvic lymphadenectomy can be associated with complications such as lymphoceles, lymphedema, and venous thrombosis, with an estimated incidence of 20% in the modern era *(22)*. An unresolved value judgment issue is whether omitting PLND during radical prostatectomy worth the risk of having occult nodal metastases go undiagnosed?

To better define the extent of PLND for prostate cancer, detailed lymphatic drainage maps of the prostate gland are needed. In contrast to penile cancer, the lymphatic drainage of prostate cancer is more complex and less well understood. In a very recent study, Mattei et al. *(23)* used multimodal fusion imaging that included single photon emission computed tomography (SPECT), computed tomography (CT), and magnetic resonance imaging (MRI), and coupled it to intraoperative lymphoscintigraphy in patients undergoing prostatectomy to map out all possible LN landing sites from the prostate gland. This study showed that majority of the landing sites are along the major pelvic vessels such as internal iliac, external iliac, and obturator regions. However, they also showed that the commonly practiced limited and extended will cover only 38 and 63% of the landing sites, respectively. The unexpected landing sites outside of the pelvic region were located along the aorta and vena cava as high as the origin inferior mesenteric artery, which constituted about 12% of total sites. A recent study by Touijer et al. *(24)* also illustrated the inadequacies of limited PLND as the odds of node positivity were greater than sevenfold in standard PLND over limited PLND. This study also demonstrated the feasibility of the laproscopic approach to perform PLND. With the advent of less-invasive surgical procedures, it would be prudent to perform PLND more frequently and extensively. By doing so, the urologic community would not only gain a better understanding of the anatomical lymphatic spread of prostate cancer but also define the true risk of nodal involvement and its biological consequences on patient outcome.

3. NONINVASIVE IMAGING APPROACHES FOR NODAL STAGING

Noninvasive imaging approaches are commonly used in the clinical evaluation of nodal involvement. Conventional imaging modalities for LN assessment include CT and (MRI), which provide high-resolution anatomical information. LNs are characterized as positive based on specific size and morphology criteria. Typically, the long-to-short axis ratio of positive nodes decreases as they become more rounded, indicating tumor infiltration. However, size criteria limits the sensitivity of CT and MRI to detect nodal metastasis due to the notion that enlarged nodes do not always correlate with tumor metastases. Inflammatory responses in diseased patients may also result in nodal enlargement, thereby obscuring the detection of metastases. Additionally, size limitations with CT and MRI can often overlook occult metastases (<5 mm), which may reduce the impact of this technique alone in nodal assessment.

Nanoparticle-enhanced lymphotropic MRI (LMRI) was recently developed and assessed for sensitivity and specificity in the detection of nodal metastases of prostate cancer *(25,26)*. Iron-oxide contrast agents are injected intravenously, where they are selectively taken up by macrophages that ultimately traffic through the lymphatics to LNs. Regions void of signal within LNs are indicative of nodal involvement as infiltrated tumor cells dislodged in these regions block access to migrating macrophages. High-resolution MRI with supermagnetic iron-oxide nanoparticles used as contrast agents for LMRI significantly improves the sensitivity of detection over conventional MRI. For instance, LMRI offers a sensitivity of positive node detection of 90% compared with approximately 35% with MRI alone *(25)*. Additionally, the incorporation of high-resolution 3D reconstruction of pelvic LNs and surrounding tissue may enable the utilization of this technique for surgical guidance *(26)*. However, LMRI is still hampered by relatively low-detection sensitivity of small nodal metastases (<5 mm) and reduced specificity due to its indirect nature. This method relies upon the inability of macrophages to traverse to LNs to infer the presence of nodal metastasis. Other nonmalignant ailments that alter the LN environment such as infection or inflammatory diseases could produce false-positive signals.

Positron emission tomography (PET) has been increasingly utilized for detection of metastatic lesions. Early studies using 18fluorine-labeled deoxyglucose (^{18}F-FDG) showed promise for detection of primary prostate tumors as well as some metastatic lesions *(27)*. However, because of the renal excretion of ^{18}F-FDG, urinal accumulation and high signal in the bladder may obscure nodal detection of prostate cancer, especially for micrometastases. Newer PET radiopharmaceuticals currently being evaluated for preoperative staging of prostate cancer pelvic LNs, such as ^{11}C-acetate *(28)* and ^{11}C-choline *(29)*, are excreted through the hepatobiliary system. Thus, these tracers circumvent the urinary signal interference issue of ^{18}F-FDG and have shown improved sensitivity for detecting nodal involvement over conventional imaging approaches *(28,29)*. The aforementioned radiotracer-directed oncologic PET imaging rely on the heightened metabolic activity of tumor cells over normal cells for detection. Like many solid tumors, the metabolic activity within a prostate tumor can be heterogeneous. Due to the heterogeneous nature and relatively low proliferative activity of prostate tumors, it would be difficult to envision uniform success in detecting metastatic lesions with current PET tracers. Further developments in personalized metabolic profiling of prostate tumors coupled with pathway-selective radiotracers will lead to more sensitive and specific PET detection of LN involvement.

Lymphoscintigraphy using ^{99m}Tc-nanocolloid particles is used extensively for lymphatic mapping of SLNs in various malignant cancers, including prostate cancer *(30)*. Radioactive colloids are injected at the peritumoral site, where they traffic to the first draining LN. The SLN is then biopsied for histological evaluation of tumor cell infiltration, which is thought to predict the overall nodal status of the patient. As noted above, the lymphatic drainage of the prostate gland is complex *(23)*, and the radiocolloid lymphoscintigraphy method provides a useful guideline to procure the likely involved nodes, and in doing so limits the extent of lymphadenectomy. Current standard procedure in prostate cancer nodal staging involves open lymphadenectomy with or without lymphoscintigraphy guidance and detailed pathological analyses of the harvested LNs. At this juncture, a one-step direct imaging approach that accurately and specifically detects nodal metastases of prostate cancer remains elusive.

4. ADENOVIRAL VECTOR-MEDIATED LYMPHANGIOGRAPHY

In designing novel particle-mediated imaging technology to query nodal status, we and others reasoned that the Ad offer some unique advantages, including its gene transfer efficiency, its large capacity to incorporate foreign genetic materials, and its innate lymphotropic properties. Ad

displays lymphotropic biodistribution in vivo and can achieve cell-specific transgene gene expression *(31,32)*. Most radionuclide lymphatic flow studies use particulate materials, such as ^{99m}Tc-sulfur colloids, ^{99m}Tc-nano- and microaggregated albumin, and liposomes to name a few *(33,34)*. When applied by interstitial directed injection, the size of the particle usually directs lymphatic uptake. For instance, particles smaller than a few nanometers usually leak into blood capillaries, whereas larger particles (about 100 nm) likely enter the lymphatic capillaries and are transported to LNs *(34)*. The optimal particle size for lymphoscintigraphy is believed to be between 50 and 200 nm *(34)*. In experimental systems, negatively charged liposomes were transported to LNs more favorably than positively charged ones *(35)*. Many historical studies documented that human adenoviruses transit to regional LNs, as this virus was originally isolated from explants of human adenoid tissue *(36)*. It is likely that human Ad size of about 70 nm and the negatively charged capsid are the reasons for its favorable lymphatic uptake. Recent studies by our group reaffirmed the lymphatic biodistribution of Ad (Fig. 1 and Color Plate 12) and its ability to mediate gene transfer to metastatic tumor cells in the LNs once transported there *(32,37)*.

To convert Ad to an imaging agent for the detection of nodal metastases of prostate cancer, a highly potent prostate-specific two-step transcriptional amplification (TSTA) system was incorporated into Ad to express imaging reporter genes. We have developed the TSTA gene expression system with the purpose of boosting the transcriptional output of specific promoters, which are often specific but weak in activity *(38)*. In the prostate-specific TSTA system, a chimeric PSA promoter was used to express a potent synthetic activator GAL4-VP16 *(39)*. GAL4-VP16 then activated high levels of reporter gene expression via a GAL4-responsive promoter in the second step (Fig. 2 and Color Plate 13). We have created TSTA Ad expressing either the bioluminescent firefly luciferase (FL and AdTSTA-FL) or the PET reporter gene-mutant herpes simplex virus type 1 thymidine kinase (HSV1-sr39tk and AdTSTA-sr39tk) *(40, 41)*. The genomic organization and the imaging activities of the TSTA Ad are depicted in Fig. 2. The AdTSTA-FL displayed 50-fold enhanced bioluminescence in prostate tumors over the conventional one-step vector, and its activity is higher than constitutive strong viral Cytomegalovirus (CMV) promoter-driven Ad *(41)*. Most importantly, not only was the TSTA Ad able to achieve a great gain in imaging signals, it retained exquisite cell selectivity, expressing only in PSA-positive and androgen receptor (AR)-positive prostate tumor cells in vivo *(40)*. In studying small animals, FL bioluminescence can be detected readily in the living mice by cooled charge-coupled device camera *(42)*. Conversely, the HSV1-sr39tk expressed in experimental tumor models can be visualized by PET scanner after injecting its positron radiolabeled substrate, F18-FHBG, into the animal *(42)*. The small animal PET studies possess several advantages over bioluminescent imaging, including its ability to produce 3D quantitative signals and its ability to translate to clinical scenarios.

To harness the unique characteristics of Ad to query prostate cancer nodal status, we combined the TSTA Ad and molecular imaging technologies and applied them in prostate tumor models that exhibited robust nodal metastasis in both subcutaneously *(43)* and prostatically *(32)* implanted tumors. By overexpressing the lymphangiogenic growth factor VEGF-C, we were able to induce consistent lymphatic metastasis in the AR + and PSA+ LAPC-9 tumor as well as in several other prostate tumor models *(43)*. The ability of TSTA Ad to produce specific imaging signals to reveal tumor-positive LNs is illustrated in Fig. 3. The forepaw and subcutaneous tumors grafted on the upper back of mice share the same regional lymphatic drainage destination of axillary LNs. Although injecting AdTSTA-FL into both forepaws should result in the transport of Ad to bilateral axillary LNs (Fig. 3a and Color Plate 14), Ad-mediated gene expression is expected to be restricted to prostate tumor cells in the involved node (i.e., axillary node ipsilateral to the implanted tumor). A specific optical signal was produced only in the ipsilateral axilla in the presence of metastases. To examine the ability of prostate-specific

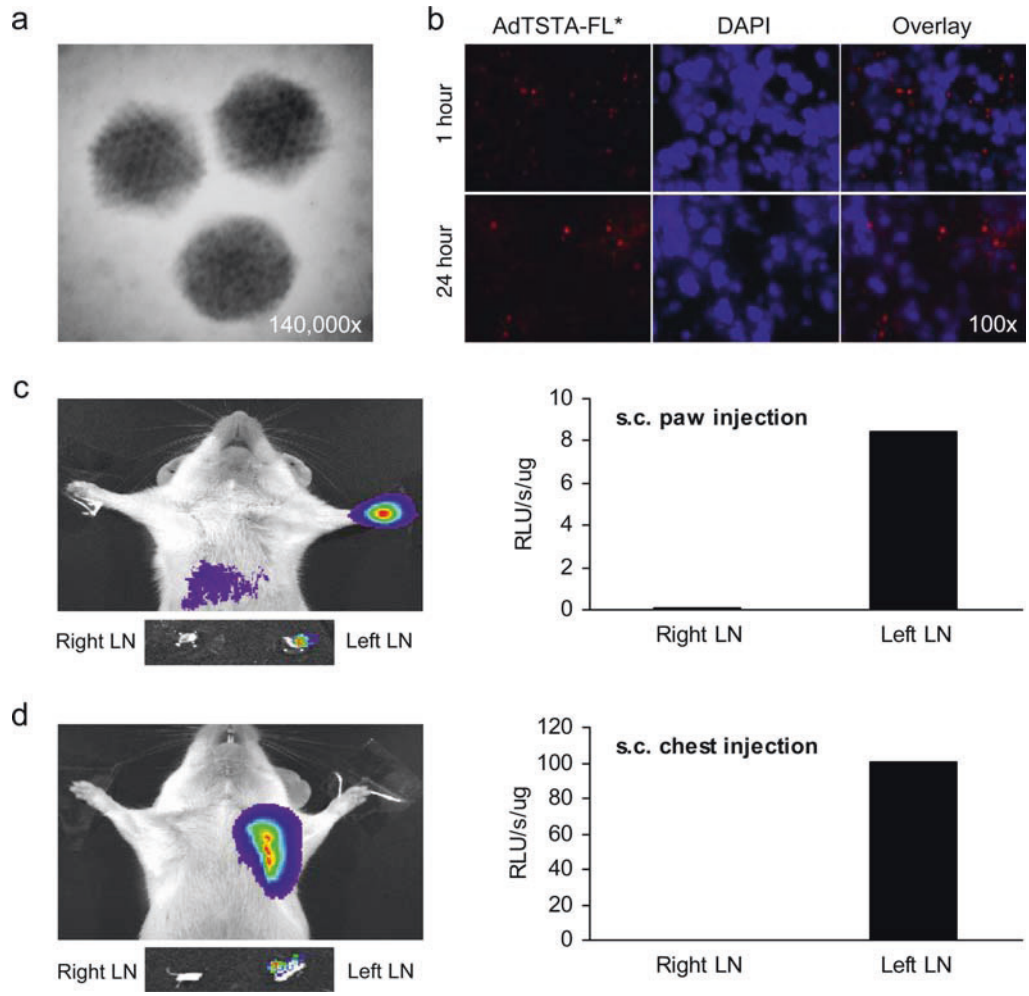

Fig. 1. Lymphotropic properties of Ad. A transmission electron micrograph image of human adenoviruses depicting the icosahedron-shaped viral particles (**a**). Alexa fluor-555 labeled AdTSTA-FL virus was injected into the forepaw of severe combined immunodeficiency (SCID) mice and confocal images of lymph node (LN) sections taken at 1 or 24 hours after viral injection revealed the presence of fluorescent virus in the draining axillary node (**b**). Functional lymphatic drainage of adenovirus from the forepaw (**c**) or the subcutaneous (s.c.) chest site (**d**) to the ipsilateral draining LN can be detected by bioluminescent luciferase signals. 1×10^7 infectious units of constitutive CMV promoter-driven virus (Ad–CMV–FL) were injected into the nontumor-bearing SCID mice. (*see* Color Plate 12)

AdTSTA-sr39tk to detect nodal metastasis by PET, we developed VEGF-C-expressing LAPC-9 tumor in a comparable experimental setting and injected the AdTSTA-sr39tk into the forepaw ipsilateral to the implanted tumor. Specific PET signals were produced in the ipsilateral axilla, which coincided with the expected sites of nodal involvement (Fig. 3c,d). Our experience indicated that peritumoral interstitial directed administration of the AdTSTA-sr39tk is more effective than extremity-directed lymphangiography to detect involved sentinel nodes as specific ^{18}F-FHBG PET signals can be produced in the occult nodal lesions only by the peritumoral injection approach *(32)*. Collectively, we showed that both large macroscopic and occult nodal lesions from subcutaneous and intraprostatic tumor models can be visualized by the TSTA Ad-mediated imaging approach *(32)*. It is the robust, yet prostate-selective expression capability of

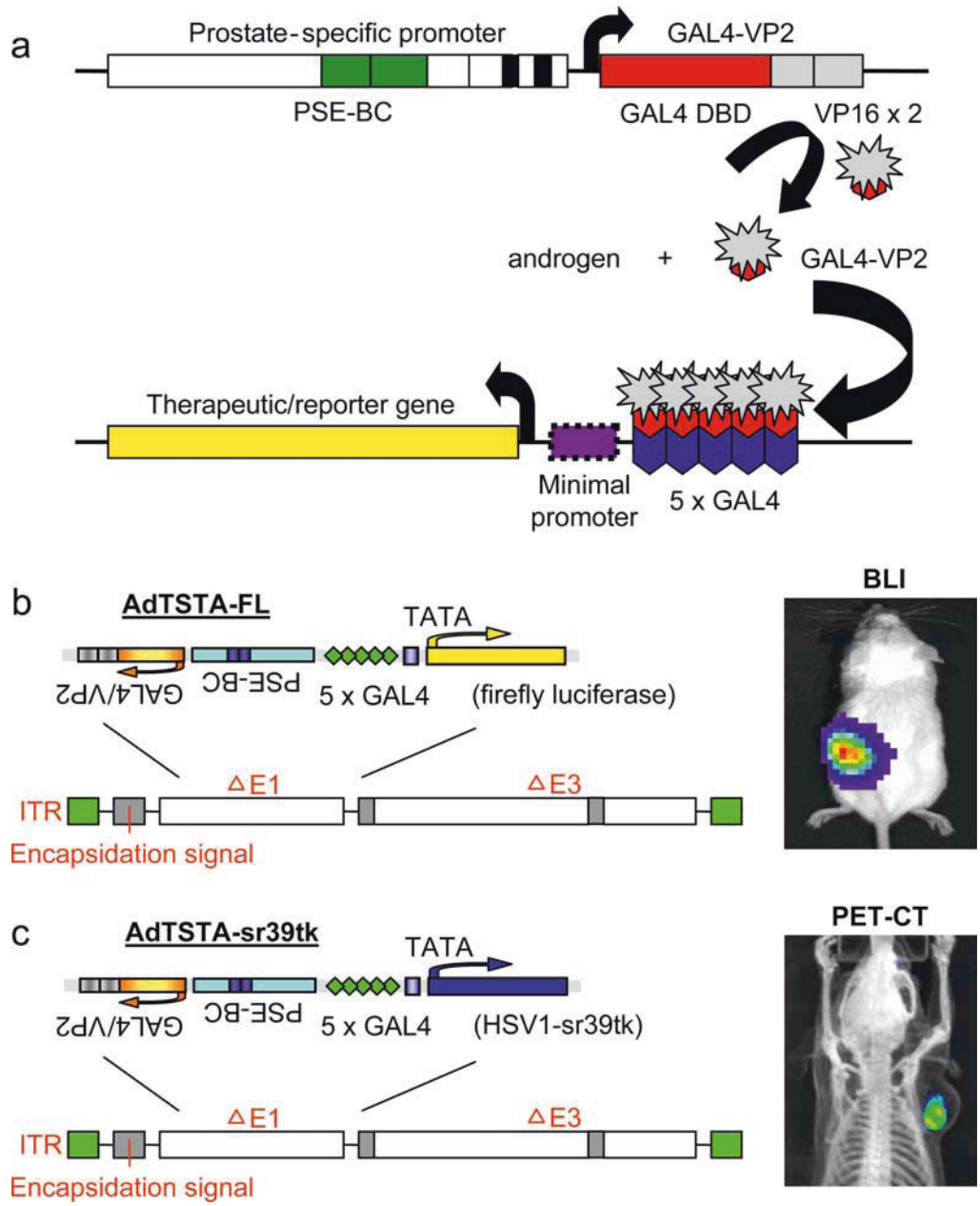

Fig. 2. Prostate-specific TSTA system and imaging reporter Ad. A schematic representation of the two-step transcriptional amplification (TSTA) system (**a**). The AdTSTA-FL and the luciferase bioluminescent signals produced by this Ad after intratumoral injection as imaged by luciferin-based bioluminescence imaging (BLI) (**b**). AdTSTA-sr39tk PET-imaging reporter vector and the ^{18}F-FHBG signal produced after intratumoral vector injection (**c**). PSE-BC = chimeric PSA enhancer/promoter; GAL4/VP2 = Fusion of the yeast GAL4 DNA-binding domain and two copies of the herpes simplex virus VP16 activation domain; TATA = TATA box; 5× GAL4 = 5 GAL4-binding sites; and HSV1-sr39tk = mutant herpes simplex virus type 1 thymidine kinase. (*see* Color Plate 13)

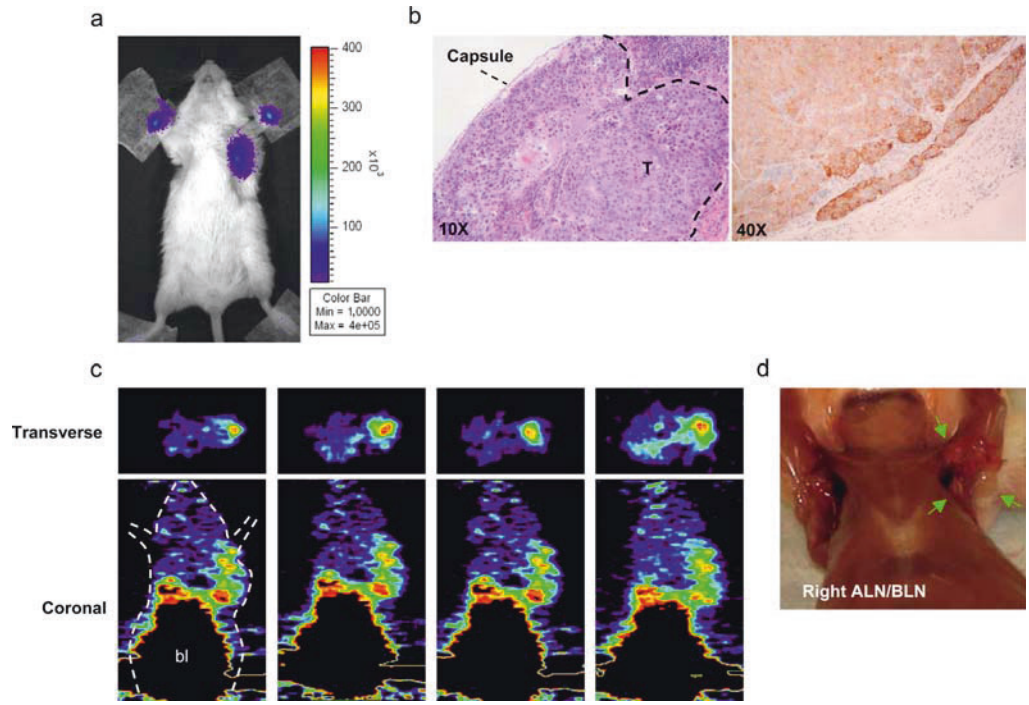

Fig. 3. Functional imaging of LN metastasis. Bioluminescence imaging of a male SCID mouse bearing LAPC-9 prostate tumor on left upper back (**a**). BLI was performed 4 days after injecting 1×10^7 infectious units of AdTSTA-FL into the left paw. Histological confirmation of metastatic prostate tumor cells in the LN by H&E (left, 10×) and anticytokeratin staining (right, 40×) (**b**). Transverse and coronal sections of 18F-FHBG PET from another LN-positive mouse scanned 4 days after injecting 1×10^8 infectious units of AdTSTA-sr39tk into the left paw (**c**). Photograph of enlarged brachial and axillary LNs (**d**). (*see* Color Plate 14)

the amplified TSTA Ad that enables direct gene transfer and imaging of nodal metastases *(32,40,44)*. The future application of this method in clinical scenarios to detect pelvic nodal metastases of prostate cancer is summarized in Fig. 4. To our knowledge this is a unique and novel approach of exploiting the lymphotropic and the selective gene expression capability of adenoviral particles to noninvasively detect nodal metastases using the clinically relevant PET imaging.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Accurate LN staging is a critical determinant for appropriate treatment strategies for cancer patients with advanced stage disease. While it remains an essential diagnostic tool for treating malignant disease, current detection methods for prostate cancer remain suboptimal. Here, we discuss several current methods for detecting prostate nodal metastasis and the specific hurdles associated with each approach. We also introduce an Ad-based gene therapy method providing a promising alternative to conventional imaging by direct mapping of nodal metastases using prostate-restricted expression of imaging reporter genes *(32)*.

Ad-mediated gene expression imaging expands the functional capabilities of current imaging approaches. First, similarities in overall particle size and lymphoid organ affinity of adenoviral

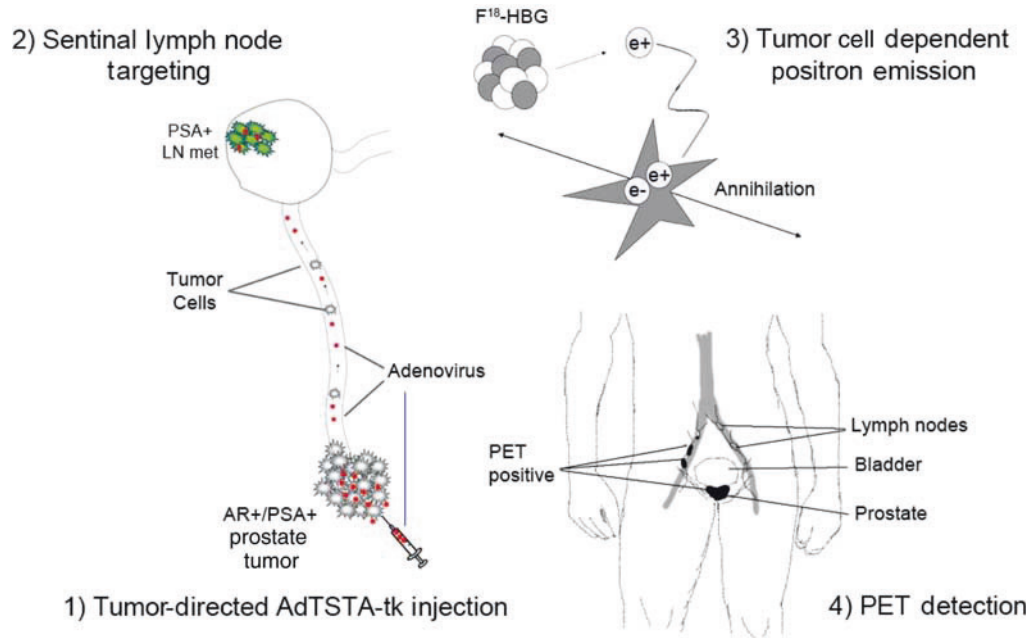

Fig. 4. Ad lymphangiography method to identify prostate nodal metastases based on gene expression. Steps for the potential clinical application of Ad-mediated detection of SLN metastases: (1) Tumor interstitial-directed AdTSTA-sr39tk injection. (2) SLN targeting: In contrast to colloid-mediated lymphoscintigraphy, Ad can transduce tumor cells within LNs. (3) Tumor cell-dependent positron emission: The gene expression control incorporated into the Ad restricts the expression of the imaging reporter gene (thymidine kinase and sr39tk) to PSA + /AR + prostate cancer cells. The expression of sr39tk leads to the trapping of 18F-HBG tracer in tumor cells in the metastatic sentinel nodes. (4) PET detection: The 18F-FHBG positron signals accumulated in tumor cells will be detected by high-resolution PET/CT.

particles and lymphotropic agents allow for comparable trafficking and function within lymphatics. Second, using prostate-restricted expression of imaging reporter genes allows specific detection of prostate tumor cells within LNs that may not possess anatomical distinction or obstruction of inflammatory cell infiltrates. Third, prostate-specific amplification of gene expression using the TSTA system augments imaging sensitivity and allows for the detection of occult nodal metastases in prostate tumor-bearing animals *(32)*.

Recent studies by our group and others showed that nodal metastases enhance and contribute to systemic metastases (i.e., lung) of prostate cancer *(43,45)*. Thus, future management of nodal metastasis would benefit by coupling diagnostic imaging of nodal metastasis to its therapeutic eradication. We and others have demonstrated the robust tumoricidal activities of the HSV-sr39tk gene and its ability to serve simultaneously as a PET reporter and a suicide gene *(44,46,47)*. Within tumor cells, HSV-sr39tk selectively phosphorylates and activates the prodrug ganciclovir, which then incorporates into DNA and ultimately leads to tumor cell death. By exploiting the dual function of HSV-sr39tk, we have demonstrated the efficacy of using ^{18}F-FHBG PET to monitor AdTSTA-sr39tk suicide treatment of primary prostate tumors *(46)*. A direct extension of this image-guided therapy will be to target and treat prostate cancer nodal metastasis.

The flexibility of Ad-based TSTA technology has allowed us to develop its utilities in two additional aspects. First, we are able to expand the coupled therapeutic/imaging capacity of the TSTA Ad by expressing up to three transgenes in a single Ad. This feat can be accomplished because (i) GAL4-VP16 activator can activate multiple GAL4-responsive genes simultaneously

and (ii) the relative large capacity of recombinant Ad can accept up to 8 kb of exogenous genes. Second, we have replaced the prostate-specific promoter of TSTA with other tissue- or tumor-specific promoters. For instance, we have demonstrated that the cancer-selective Mucin-1 promoter-driven TSTA can achieve over 200-fold augmentation of transcriptional activity over a one-step approach. The Muc1-TSTA Ad imaging vector can specifically target breast tumors, including nodal metastases of breast cancer *(48)*. Collectively, the functional gene expression-based imaging that we have developed with the recombinant TSTA Ad represents a novel technique to specifically target and map nodal metastases. Here, we have demonstrated the feasibility of this approach for prostate cancer. We believe this technology could be a template for developing effective image-guided therapy for not only prostate but also other types of carcinoma.

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V Therapeutic Targeting of the Lymphovascular System

17 Therapeutic Targeting of the Lymphovascular System in Cancer: Promise and Challenge

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Contents

ABSTRACT

Acquisition of the metastatic phenotype by cells within a solid tumor initiates a sequence of events that represent, for most cancer patients, the primary cause of morbidity and mortality of this disease. This realization gave birth to a field of research focused on unraveling the nature of the metastatic process and led to many groundbreaking scientific achievements. However, it has proven extremely difficult to translate these discoveries into efficacious treatments that target the metastatic process. While hematogenous dissemination of tumor cells through blood vessels is well understood, the contribution of lymphatic metastasis to the overall metastatic process is more controversial. We attempt to define the common ground that must be achieved by basic scientists, translational researchers, and clinicians before targeting of lymphatic metastasis can be attempted in the clinic. To better illustrate the key issues involved in this debate, we examine the controversies related to tumor dissemination through lymphatic vessels from two distinct points of view. First, we review the clinical data regarding invasive melanoma, a tumor with propensity for lymphatic metastasis as the initial route of metastasis. Second, we examine how the uncertainty regarding the clinical relevance of lymphatic metastasis affects the plans for clinical development of antibody antagonists of the vascular endothelial growth factor receptor-3

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(VEGFR-3), an important mediator of tumor lymphangiogenesis. Our goal is to provide a framework for ongoing dialog in the field.

Key Words: lymphatic metastasis; lymphangiogenesis; melanoma; targeted therapy; monoclonal antibody; VEGFR-3

1. INTRODUCTION

A tumor is a solid, cellular growth that arises from normal tissue due to deregulation of cellular growth control mechanisms and the failure of apoptotic safety mechanisms *(1)*. With the exception of cancers of the brain and the hematologic system, tumors rarely cause death while restricted to their tissue of origin. The acquisition of the malignant phenotype, typically a result of an accumulation of mutations in the genome of the cancer cell over a period of years, allows malignant cells to escape from the local microenvironment of the primary tumor, invade neighboring tissues, and ultimately disseminate to distant organs *(2)*. The process of systemic tumor dissemination has been termed "metastasis," a word of Greek origins combining the concepts of "change" (meta) and "standing" (stasis). Thus, in its metastatic form, cancer is a disease caused by transformed cells that no longer "stand still." Acquisition of the metastatic phenotype leads to crucial changes in the basic biology of tumors and represents, for most cancers, the primary cause of morbidity and mortality for patients with solid tumors. In turn, this realization prompted the birth and rapid growth of an entire field of research focused on unraveling the nature of the metastatic process. Despite enormous expenditures by funding agencies and groundbreaking scientific achievements, the most efficacious treatment for most cancer patients remains the detection and timely surgical removal of the tumor while it is still confined to the primary site.

Tumor cells escape from the local microenvironment and disseminate via two principal routes: vascular (hematogenous) and lymphatic. Hematogenous dissemination involves the invasion of blood vessels by tumor cells and is greatly facilitated by the development of tumor-associated vasculature, a process termed tumor angiogenesis *(3)*. The role of this process in the seeding of tumor to distant organs is well-established. The important role of lymphatic metastasis in the clinical behavior of many solid tumors (e.g., melanoma, breast, prostate, and head and neck cancers) is also well-established *(4–9)*. In contrast, the contribution of lymphatic metastasis to the overall metastatic process is less well-defined and, from the perspective of some investigators, more controversial (reviewed in *[10, 11]*). As illustrated below, rather than being a purely academic matter, the controversy regarding the importance of lymphatic metastasis impacts both clinical practice and the development of therapeutic modalities that may be used to inhibit this form of metastatic disease.

It is beyond the scope of this chapter to provide a detailed review of metastasis or to describe the mechanisms that mediate lymphatic metastasis. These subjects have been covered in depth in this volume and elsewhere *(12–14)*. Rather, we will focus on illuminating the common ground that must be identified among basic scientists, translational researchers who study lymphatic metastasis, and clinicians who treat patients, before targeting of lymphatic metastasis in the clinic can become a reality. To this end, we first examine some of the salient controversies and challenges related to tumor dissemination through lymphatic vessels (i.e., lymphatic metastasis) and further explore these issues using invasive melanoma—a potentially aggressive tumor with propensity for lymphatic metastasis as a first site of spread—as a paradigm for additional discussion. In an attempt to provide a framework for the discussion as it relates to development of a potential therapeutic modality, we will then examine how the overall uncertainty regarding the clinical relevance of lymphatic metastasis as a therapeutic target impacts plans for the clinical development of novel monoclonal antibodies (mAbs) that act as antagonists of the VEGFR-3 (see below). Although we are aware that this approach may raise as many questions as provide answers, our goal is to provide a framework for ongoing dialog in the field.

2. THERAPEUTIC TARGETABILITY OF THE OVERALL METASTATIC PROCESS

The noun "metastasis" has two usages: the process of dissemination of malignant cells from the primary tumor and (in the inflected plural form of "metastases") the secondary or tertiary tumor in a different anatomic location derived from such a process. In the context of this discussion, antimetastatic therapy of any kind refers to the first usage, that is, this form of therapy attempts to inhibit the process of tumor dissemination. Controversy among clinicians still remains regarding what, if anything, can be done to inhibit this process.

For illustrative purposes, let us assume the following hypothetical clinical situations wherein at the time of primary tumor diagnosis, there exist only three distinct scenarios with respect to the "metastatic state" of the tumor. In the first scenario, no dissemination has occurred and the patient is cured by surgical resection of the primary tumor. In the second scenario, disseminated tumor cells already exist in the form of undetectable (occult), micrometastases either in target tissues or in circulation (reviewed in *[15]*). A similar but distinct situation occurs when metastasized tumor cells exist, often in a dormant state that may persist for many years, following the initial course of treatment. This condition is sometimes referred to as minimal residual disease (MRD) *(16)*. A third scenario may exist when metastases to distant organs are evident at the time of diagnosis. In many cases, it may be impossible to distinguish between the first two scenarios and as a consequence, adjuvant therapy may be offered to the patient, consisting of chemotherapy, targeted treatment with biologics, or both, to minimize the possibility that dormant tumor cells reenter the cell cycle and develop into clinically relevant metastases with potentially dire consequences to the patient *(17)*. Careful attention to the aforementioned definitions of "metastasis(es)" is critical to best understand this discussion. In this context, adjuvant therapy is not a form of antimetastatic therapy, because it targets the survival or growth of disseminated tumor cells (metastases) and not the *process* of dissemination (metastasis).

The above discussion thus leads to the following crucial question: Is it too late at the time of diagnosis and surgical resection of the primary tumor to initiate antimetastatic therapy? Assuming that dormant tumor cells exist, the answer necessarily depends on the anatomic location of MRD. Using melanoma as an example, if all or most of the potentially clinically relevant residual disease resides in distant target organs (e.g., liver, brain, and lungs), then antimetastatic therapy of any kind is unlikely to be successful. If, on the other hand, a significant proportion of residual disease is regional (e.g., "intransit" melanoma metastasis and/or regional lymph node metastasis, both clinically relevant forms of intralymphatic dissemination of melanoma), antimetastatic therapy may be contemplated in order to prevent subsequent metastatic dissemination to distant organs or even additional regional disease.

3. CLINICAL SIGNIFICANCE OF LYMPH NODE METASTASIS

Superimposed on the overall discussion of the "targetability" of the metastatic process in general, there is a more focused controversy regarding the importance of lymphatic metastasis in relation to the establishment of distant metastases. There are two schools of thought on this issue *(18)*. The first, termed the "marker" or "dead end" hypothesis, states that the presence of tumor cells in the regional lymph nodes draining the primary tumor is nothing more than a biomarker for the acquisition by cells in the primary tumor of a metastatic or "invasive" phenotype. Proponents of this hypothesis reason that the structure of lymphatic capillaries is optimized for uptake of macromolecules and entry of immune cells; therefore, malignant cells easily invade these lymphatic vessels. Once in the lymphatic capillaries, they maintain, tumor cells are passively transported to the lymph nodes through collecting lymphatic vessels together with lymph and

leukocytes. An implication of the marker hypothesis is that invasion of lymphatic vessels within or in proximity to the primary tumor is not, in most cases, clinically relevant with respect to cancer mortality.

An alternative hypothesis, termed the "incubator" or "staging area" hypothesis, maintains that regional lymph nodes colonized by tumor cells can act as either an incubator for dormant tumor cells or a staging area for secondary dissemination to distant organs. From this perspective, invasion of the lymphatic capillaries and the proximal (i.e., regional) draining lymph nodes constitutes an important and early phase of the overall metastatic cascade. Accordingly, in the secondary phase of metastasis, regionally metastatic lymph nodes serve as a staging area for further dissemination of the tumor to distant organs. This can occur either by invasion of blood capillaries of the lymph nodes or by the migration of tumor with the normal flow of the lymph through the collecting lymphatic vessels to the principal thoracic ducts and then into the systemic circulation *(18)*. There is a significant clinical experience suggesting that melanoma metastasis progresses, in large part, according to the "incubator" model.

Ultimate resolution of these hypotheses—"marker" versus "incubator"—is further complicated by the observation that, despite common themes in the overall metastatic process across a spectra of solid tumors, significant differences in the underlying biology of the metastatic cascade among such tumors confound attempts to provide a simple, unified model. The strength of clinical evidence supporting either the "marker" or the "incubator" hypothesis was extensively discussed during the Second International Symposium on Cancer Metastasis and the Lymphovascular System (San Francisco, May 2007) on which the present volume is based. Although a resolution of this controversy was not achieved, there was a general agreement that the controversy impacts the advancement of cancer treatment on two distinct fronts: (1) clinical practice and (2) the development of targeted biologic therapies.

4. IMPACT OF THE "MARKER" VERSUS "INCUBATOR" CONTROVERSY ON CLINICAL PRACTICE

Using melanoma as an example, it is evident that the clinical relevance of regional lymphatic metastasis is well understood. Not only does lymphatic metastasis represent the most common first site of spread in patients with melanoma, its presence is associated with increased risk of subsequent distant failure and death from melanoma *(9)*. Identification of regional lymphatic metastasis in patients with clinically *uninvolved* regional lymph nodes but in whom the primary melanoma is considered to be associated with a relevant risk of occult metastatic disease is widely established as a clinically useful endpoint. As such, surgical evaluation and pathologic staging of regional nodal basins at risk—using the now well-established technique of intraoperative lymphatic mapping and sentinel lymph node biopsy—are used to identify sentinel lymph nodes (i.e., the nodes that receive afferent lymphatic drainage from the primary tumor) and represent those most likely node(s) to contain metastatic disease, if any are involved *(9, 19–21)*. The significance of the sentinel node is such that the presence of microscopic nodal metastasis is the most important independent predictor of recurrence and survival among patients with early-stage melanoma *(20)*. Early identification of nodal disease also addresses other important issues in the management of patients with melanoma. These include (1) improved pathologic nodal staging to identify patients who may benefit clinically from more aggressive regional lymph node surgical therapy, to identify patients who may benefit from adjuvant therapy, and to better stratify patients in adjuvant therapy trials to better determine who benefits from such treatment; (2) the ability to achieve regional node control; (3) a possible survival benefit in a subset of patients; and (4) the desire to implement and utilize a clinical approach that is minimally invasive

when possible. Importantly, while the presence of clinically evident regional metastases or other intralymphatic manifestations of melanoma may be associated with significant morbidity (e.g., lymphedema related to impaired lymph flow to the affected regional nodal basin and associated potentially debilitating soft tissue swelling, particularly of an extremity), extensive surgical resection of regional lymph nodes (e.g., formal lymph node dissection for many solid tumors) can also severely impair lymph flow in the affected tissue and result in lymphedema. Such "secondary" lymphedema may occur in the ipsilateral arm following axillary lymph node dissection for breast cancer or melanoma and in the lower extremities after groin lymph node dissection, for example, following surgery for melanoma or cervical cancer *(22, 23)*.

To further illustrate using melanoma as an example, extensive regional lymphadenectomy would only seem sensible from a "curative" standpoint if the regional nodes represent the only evidence of metastatic disease. If the incubator hypothesis is correct, these regional nodes serve as conduits for distant organ spread; as such, there may be a survival benefit associated with their removal. While this is still somewhat contentious among some clinicians based on their attachment to early, likely underpowered, randomized clinical trials in melanoma demonstrating no overall survival benefit among patients with clinically uninvolved nodes who had elective regional lymph node dissection compared with patients who underwent nodal observation alone, there is evolving and compelling evidence from an international ongoing randomized clinical trial (Multicenter Selective Lymphadenectomy Trial [MSLT-1]) that early surgical intervention of microscopic regional metastases not only achieves durable regional nodal control, but may also confer a survival advantage compared with nodal observation followed by regional lymphadenectomy performed only when the regional disease becomes clinically detectable *(21, 24, 25)*.

In the breast cancer arena, researchers studying women diagnosed with breast cancer using data from the Surveillance, Epidemiology and End Results (SEER) program reached a similar conclusion (and in contrast to earlier studies *[26]*) that the risk of death was significantly reduced in women who underwent lymphadenectomy, presumably due to removal of undetectable micrometastases within the draining lymph nodes *(27)*. The widespread use of sentinel lymph node biopsy in breast cancer to identify those patients with occult metastatic regional lymph node disease also supports this hypothesis in clinical practice *(28–30)*.

5. TARGETING LYMPHANGIOGENESIS AS ANTIMETASTATIC CANCER THERAPY

Recent advances have permitted significant unraveling of the basic biology of lymphatic vessels as well as the induction and local expansion of the lymphatic capillary bed by the primary tumor (see earlier chapter by K. Alitalo). At the same time, increased understanding of the lymphatic metastatic cascade has emerged from the evaluation of specimens obtained from cancer patients. Retrospective studies using archival tumor specimens have clearly established increased lymphatic vessel density (LVD) in the peritumoral area as a strong predictor of tumor metastasis to regional lymph nodes (reviewed in *[31]*). In turn, metastasis to regional lymph nodes is a significant adverse predictor of clinical outcome for patients with a majority of solid tumors (reviewed in *[32]*). In melanoma, this relationship is particularly noteworthy; in at least one study, LVD was used to estimate patient prognosis *(33)*. Increase in LVD occurs through the process of lymphangiogenesis, that is, the sprouting and maturation of new lymphatic capillaries from preexisting lymphatic vessels and is strongly stimulated by the production of lymphangiogenic growth factors by the tumor cells, tumor stroma, or infiltrating leukocytes *(34)*.

Concerted research efforts to elucidate the basic biology of lymphatic vessels have led to the identification of a number of ligands and their cognate receptors that regulate the process of lymphangiogenesis (reviewed in *[35]*). Two members of the vascular endothelial growth factor (VEGF) family, VEGF-C and VEGF-D, are known to mediate powerful lymphangiogenic signaling in lymphatic endothelial cells (LECs) by activating the receptor tyrosine kinase (RTK) VEGFR-3 (reviewed in *[13]*). Initial preclinical studies that established the importance of VEGFR-3 for lymphangiogenesis in vivo and, in turn, for lymphatic metastasis of tumors, utilized a "trap" approach in which the soluble extracellular portion of VEGFR-3 was expressed in transgenic mice or using viral vectors *(36, 37)*. More recently, a rat antimouse monoclonal antibody mF4-31C1 that binds to and antagonizes mouse VEGFR-3 was developed and has been shown to powerfully inhibit lymphangiogenesis in a murine wound-healing model *(38)*. mF4-31C1 has been used in a number of murine models of human disease as a "proof of concept" mAb—that is, to demonstrate in vivo utility that might be achievable in patients with mAbs to human VEGFR-3. Studies thus far have addressed the role of lymphangiogenesis in models of corneal and pulmonary inflammation and in the expansion of the lymphatic vasculature in the lymph node during the normal immune response *(39–41)*. More importantly, in the context of the present discussion, blockade of VEGFR-3 with mF4-31C1 has been demonstrated to strongly inhibit tumor metastasis to regional lymph nodes and to distant organs *(42, 43)*. The extent to which these findings are relevant to situations encountered in clinical practice is of critical importance with respect to eventual efforts to inhibit lymphatic (and overall) metastasis in cancer patients and is further elucidated in the next section.

6. POSSIBLE CLINICAL UTILITY OF ANTAGONISTS OF VEGFR-3 SIGNALING

One of the authors of this chapter (B.P.) directed the development of antagonist mAbs to VEGFR-3 at ImClone Systems, a biotechnology company in New York. This task was not undertaken with the primary goal of developing a therapy for lymphatic metastasis. Rather, VEGFR-3 emerged as a potential drug target because of the observations that tumor blood vessels express this RTK, while its expression in adults is normally restricted to lymphatic endothelium and to highly fenestrated vascular endothelium *(44)*. Tumor angiogenesis is thought to be largely mediated by activation of VEGFR-2 on the endothelium of tumor blood vessels by excess VEGF produced by tumor cells and tumor stroma *(34)*. We reasoned that expression of VEGFR-3 in tumor blood vessels might provide a proangiogenic signal in response to VEGF-C and VEGF-D that would complement the signaling through the VEGF–VEGFR-2 axis. In fact, monotherapy with mF4-31C1 has been shown to lead to a statistically significant reduction in tumor volume in a number of xenograft tumor models, and this activity was accompanied by a reduced blood vessel density in the xenografts *(34)*. Thus, the primary justification for possible clinical trials of mAb antagonists of human VEGFR-3 is that these mAbs may augment antiangiogenic activity of therapeutics that target either VEGF or VEGFR-2 or that it might be active in situations when such therapies lose efficacy.

These considerations notwithstanding, it did not escape our attention that treatment of cancer patients with an antagonist mAb to VEGFR-3 may lead to significant reduction of lymphangiogenesis at the primary tumor, at metastatic sites, or within draining lymph nodes.

What can we learn from studies in which tumor lymphangiogenesis was targeted with the "proof of concept" mAb mF4-31C1 that might encourage clinical studies of inhibitors of lymphangiogenesis in cancer patients? First, in an orthotopic model of breast cancer metastasis to lymph nodes and to lung, both lymphatic and pulmonary metastases were significantly inhibited by blockade of VEGFR-3 with mF4-31C1 *(42)*. Taken together with other recent

data, these studies provide some of the first experimental support for the "incubator" or "staging area" hypothesis of metastasis *(45)*. Second, in the above model and in recent experiments using a prostate carcinoma model of lymphatic metastasis, VEGFR-3 blockade was superior to VEGFR-2 blockade in inhibiting lymphatic metastasis despite the observation that blockade of VEGFR-2 led to greater reduction in the volume of the primary tumor in both studies *(42, 46)*. VEGFR-2 is present in endothelia of both vascular and lymphatic capillaries, and the activation of this RTK has been implicated in tumor-induced lymphangiogenesis *(47)*. Thus, results of two independent studies indicate that VEGFR-3 may be the preferred target in inhibiting tumor lymphangiogenesis and lymphatic metastasis.

The above successes notwithstanding, several salient issues arise with respect to the above-mentioned studies with mF4-31C1. First, successful inhibition of lymphatic and distant organ metastasis depends critically on the timing of VEGFR-3 inhibition. Thus, antilymphangiogenic therapy with mF4-31C1 potently blocks lymphatic metastasis, when the mAb treatment is initiated soon after the implantation of the tumor. Unfortunately, opportunities to translate experimental intervention at this stage of tumor growth into clinical practice are rare. Specifically, delay in the administration of the mAb in the so-called "intervention" model led to significant diminution of the antimetastatic efficacy of the mF4-31C1 treatment *(42)*. Second, xenograft models that utilize cell lines derived from human tumors implanted into immunodeficient mice are rather poor at mimicking the course of metastatic disease in humans. In particular, the cell lines are usually chosen for rapid growth in vivo and for high rates of metastasis that typically occurs within weeks of tumor implantation. In humans, progression of cancer from a local tumor to one that has metastasized may occur over a period of months to years. Furthermore, the phenotype of tumor cells used in mouse models is a result of an accumulation of chromosomal changes and mutations acquired in the cancer of origin and during the subsequent adaptation to culture. In contrast to the typical process of malignant progression in humans, major genetic changes only rarely occur in xenograft mouse models during the rather short time necessary for the primary tumor to metastasize.

Taken together, it is extremely difficult to extrapolate the data obtained with mF4-31C1 in preclinical murine models of lymphatic and distant organ metastasis to predict the efficacy of antilymphangiogenic treatment of cancer patients with mAbs to VEGFR-3. These considerations highlight both the paucity of satisfactory murine models of occult and residual disease and the significant challenge involved in studying cancer cell dormancy in general. The absence of such models explains, at least in part, the reluctance of clinical investigators and biopharmaceutical companies that develop cancer therapeutics to contemplate trials of antimetastatic therapy.

Should anti-VEGFR-3 mAbs advance to phase II clinical trials, it seems unlikely, based on the uncertainties detailed in the above discussion, that the initial efficacy trials will involve attempts to control the process of metastasis. More likely, the clinical studies will focus on the reduction of growth of metastatic tumors. Despite these significant challenges, there are several reasons why the difficulties in studying metastasis in general and lymphatic metastasis in particular should not preclude an optimistic conclusion to our analysis. In general, when cancer therapeutics show signs of efficacy in early phase II clinical trials (usually conducted in patients with advanced, difficult to treat cancers), their use is typically expanded to include the adjuvant therapy setting. Patients who receive adjuvant therapy usually have an earlier (and often more "treatable") stage of disease, have treatment initiated earlier, and continue it for a longer term. Adjuvant trials may reveal a potential of a drug (e.g., mAb to VEGFR-3) to inhibit metastatic spread. It may also be possible that performing initial phase II studies on patients with tumor types in which locoregional involvement is particularly important may reveal a benefit of the inhibition of lymphangiogenesis for locoregional control as well as for the reduction of tumor metastasis to distant organs.

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18 Comparison of Liposomal and Aqueous Blue Dye in Visualization of Lymph Nodes Prior to Lymphadenectomy

Peter Hirnle, MD

Contents

ABSTRACT

Intraoperative identification of regional lymph nodes is massively improved by their effective preoperative staining, which is necessary for the selectivity of sentinel node lymphadenectomy. Currently used aqueous dye solutions, such as Patent Blue V (PBV), do not reliably provide the necessary properties regarding intensity and duration of contrast.

To find a better substance for preoperative staining, a study was designed, in which conventional PBV in aqueous solution and PBV encapsulated in liposomes were compared in terms of their staining properties.

An extrusion technique using membranes with a thickness of 0.2, 0.4, and 5 μm supplied lecithin–cholesterol liposomes in a molar ratio of 3:1. For the experiment, eight female pigs with an average weight of 40 kg were used. Each pig was administered a 0.5-ml depot containing 12.5 ± 0.2 mg of aqueous PBV into each of the four upper and lower mammary glands on the left side. On the right side, the same dosage of PBV encapsulated in liposomes was injected.

After 3, 6, 12, and 24 hours stained lymph nodes of the neck, pelvis, and groin were excised and photographically documented. Their respective PBV concentrations were additionally analyzed by spectrophotometry. It was found that in each case the intensity and duration of staining were better in case of liposomal dye. It is, therefore, recommended to prefer PBV encapsulated in liposomes for preoperative staining and the identification of sentinel lymph nodes.

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Key Words: lymph node staining; sentinel node; liposomes

1. INTRODUCTION

Lymph node metastases are an important prognostic factor in cancer. To improve outcome, the areas draining malignant tumors can be completely removed. Radical surgery usually involves the removal of all regional lymph nodes, but also of their surrounding tissue and of lymph vessels. This poor selectivity causes side effects, such as secondary lymphedema. This problem can only be overcome by preoperative staining of lymph nodes, which allows their selective removal. Investigations targeting this problem were performed already 20 years ago *(1)* and were called later "sentinel node technique."

Preoperative staining of lymph nodes can be carried out with different dyes *(2–4)*, radioactivity *(5,6)*, or with a combination of both *(7,8)*.

In many recently published reports, blue staining and radionuclide labeling of lymph nodes were performed. Both techniques were used in a wide range of tumors. Most reports are focused on colorectal carcinoma, including one experimental study *(9)* and different clinical studies *(10–15)*. There are also many reports concerning carcinoma of the breast *(16–21)*, and cervical carcinoma *(22–26)*. Other reports refer to gastric carcinoma *(27,28)*, melanoma *(29,30)*, thyroid carcinoma *(31)*, vulvar carcinoma *(32)*, or head and neck tumors *(33)*.

The comparison between blue dye alone versus blue dye and radioactive labeling *(17–19,21–25,27)* demonstrated that the combined technique is significantly superior. As a possible explanation, some authors *(25)* suggested that the blue dye remains too short in the lymph nodes and cannot be detected in sufficient amounts already after 30 minutes and is completely gone 50 minutes after interstitial injection. This short time, in which the blue staining is available, is probably the reason why other authors *(28,31)* do not recommend to use dye and prefer radioactive labeling only.

To explore the properties of blue dye preparation following different pharmacokinetics, the comparison between aqueous (conventional) and liposomal (novel) dye after interstitial injection was performed in this study *(34)*. The hypothesis had to be proven that the disadvantages of blue dye will vanish after incorporating it into the liposomes. In this case, radionuclide labeling would concur with a new technique, which has two advantages: the lymph nodes are visible with the naked eye and no radioactive contamination occurs.

2. MATERIAL AND METHOD

Mammary glands have a highly developed and well-known lymphatic drainage system. For the subsequently described experiment, it was hypothesized that indirect lymphography of mammary glands with liposomes loaded with blue dye would lead to improved staining of regional lymph nodes compared with indirect lymphography with an aqueous solution of the same dye.

The studied animals were eight female pigs with an average weight of 40 kg. The injections were performed into the four upper and lower mammary glands.

On the left side, each pig received a 0.5-ml depot containing 12.5 ± 0.2 mg of aqueous PBV. On the right side, the same dosage of PBV encapsulated in liposomes was injected.

The liposomes were produced by extrusion using membranes with a thickness of 0.2, 0.4, and 5 μm. The resulting liposomes were built of lecithin and cholesterol in a molar ratio of 3:1. The liposomes had an average diameter of 172 nm. The total lipid concentration was 41 mmol/l.

At different time intervals with the first pig after 3 hours, two more after 6 hours, four after 12 hours, and the last one after 24 hours, the pigs were put under general anaesthesia. Using the technique of operative laparoscopy, the excision of the lymph nodes in the pelvis was performed. After this procedure, the pigs received an overdose of anesthetics. The lymph nodes of the groin and neck were removed, photographed, and weighed. Additional histological workup was performed on representative parts of the lymph nodes after the specimens were immersed into ethanol and minced to a homogenous cell suspension. The resulting suspension was centrifuged for 30 minutes at $1000 \times g$. Spectrophotometry revealed PBV concentrations of 635 nm in the specimen.

3. RESULTS

The laparoscopic removal of pelvic lymph nodes took 35 ± 12 minutes. The complete removal of stained lymph nodes was possible. This was confirmed by the investigation of the retroperitoneal space after the animals were killed.

Three hours after the injection of the aqueous and the liposomal preparation of PBV, the lymph nodes of the groin and the pelvis were well-stained bilaterally. The neck lymph nodes on the right (= liposomal) side showed good contrast and could, therefore, be easily identified. On the left (= aqueous) side, the contrast was much weaker, and the identification of lymph nodes was consequently more difficult. A 4.55 times higher concentration of PBV was found in lymph nodes, where the liposomal solution had been injected (Fig. 1A, B).

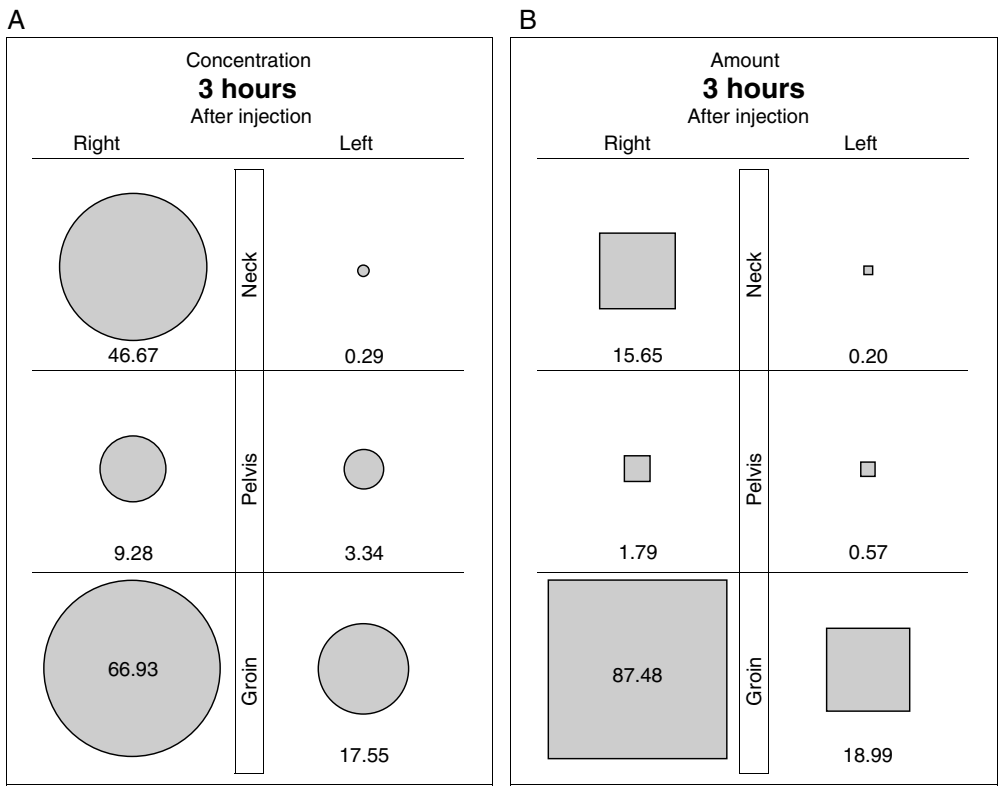

Fig. 1 (A) Concentration of Patent Blue in lymph nodes of the neck, the pelvis, and the groin 3 hours after injection. (B) Amount of Patent Blue in lymph nodes of the neck, the pelvis, and the groin 3 hours after injection.

Six hours after the injections, the staining of neck lymph nodes on the aqueous side was not visible any more. In contrast, the lymph nodes on the right side of the neck were still detectable. The extracted amount of PBV was 12.86 times higher on the liposomal side than on the aqueous side (Fig. 2A, B).

Twelve hours after the injections, the neck lymph nodes of the liposomal side remained visibly stained, whereas those on the aqueous side were not stained any more. The extracted amount of PBV was 10.03 times higher on the liposomal side than on the aqueous side (Fig. 3A, B).

After 24 hours, no staining of the neck lymph nodes was observable. The pelvic lymph nodes were stained only poorly on both sides. In contrast, the lymph nodes in the groin were still visible only on the liposomal side. The extracted amount of PBV was 4.60 times higher on the liposomal side than on the aqueous side (Fig. 4A, B).

These results demonstrated clearly that with the liposomal preparation much more blue dye could be introduced into the lymph nodes than by using the aqueous solution (Fig. 5).

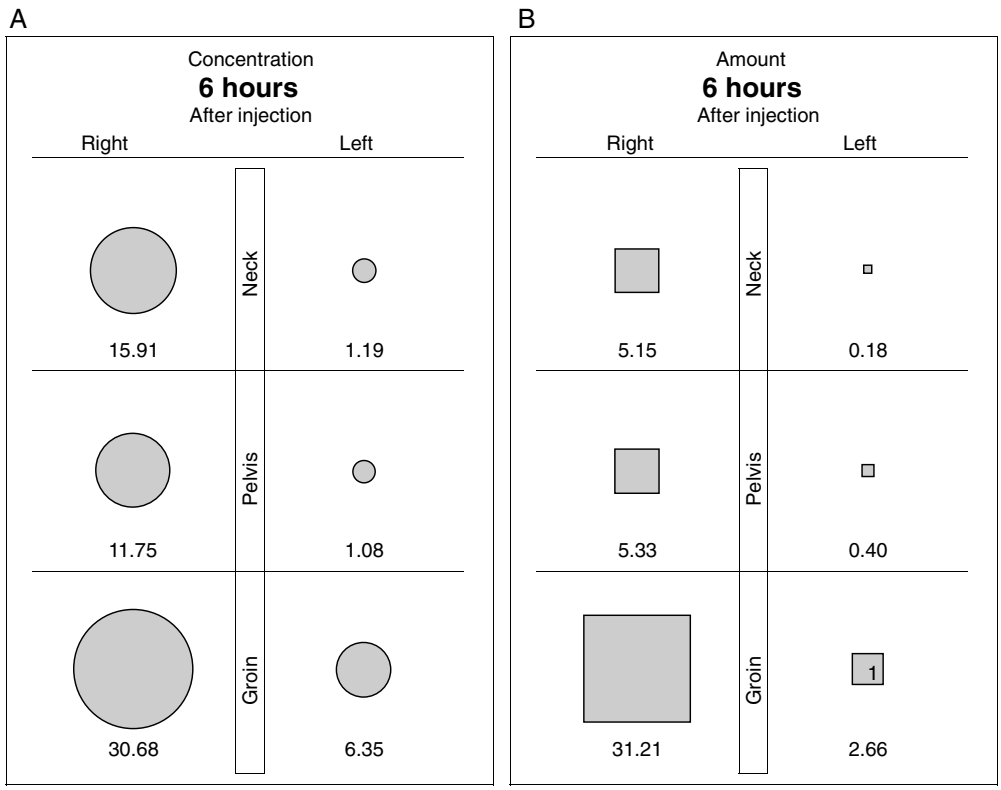

Fig. 2 (A) Concentration of Patent Blue in lymph nodes of the neck, the pelvis, and the groin 6 hours after injection. (B) Amount of Patent Blue in lymph nodes of the neck, the pelvis, and the groin 6 hours after injection.

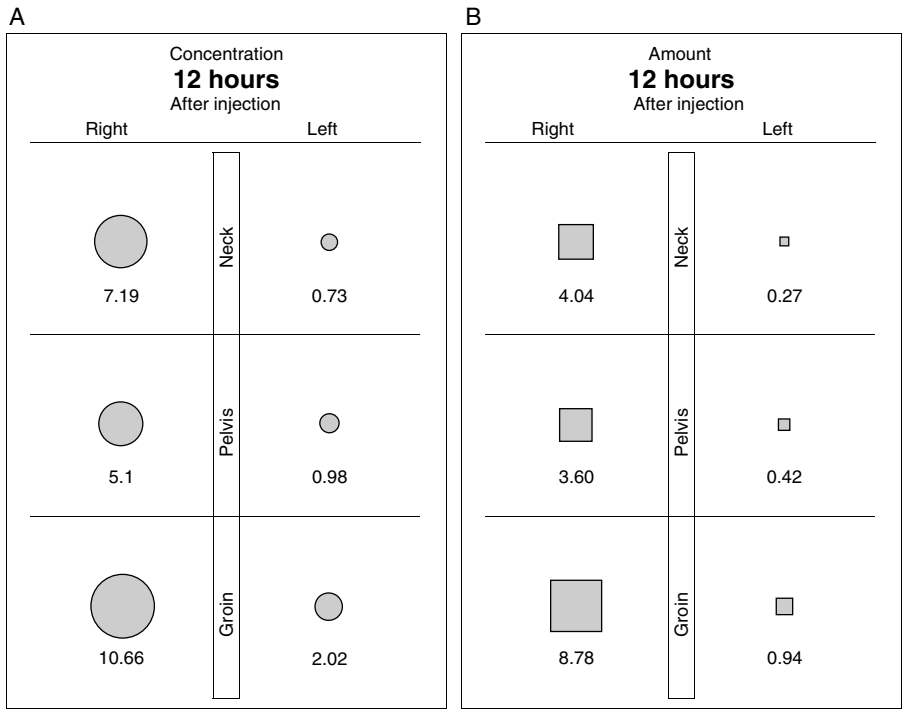

Fig. 3 (A) Concentration of Patent Blue in lymph nodes of the neck, the pelvis, and the groin 12 hours after injection. (B) Amount of Patent Blue in lymph nodes of the neck, the pelvis, and the groin 12 hours after injection.

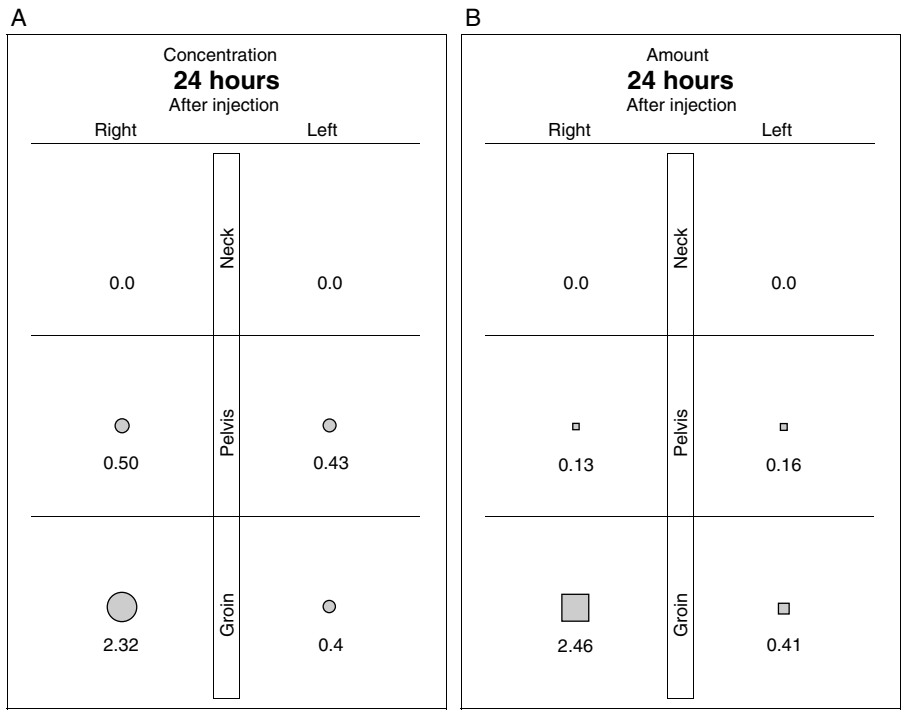

Fig. 4 (A) Concentration of Patent Blue in lymph nodes of the neck, the pelvis, and the groin 24 hours after injection. (B) Amount of Patent Blue in lymph nodes of the neck, the pelvis, and the groin 24 hours after injection.

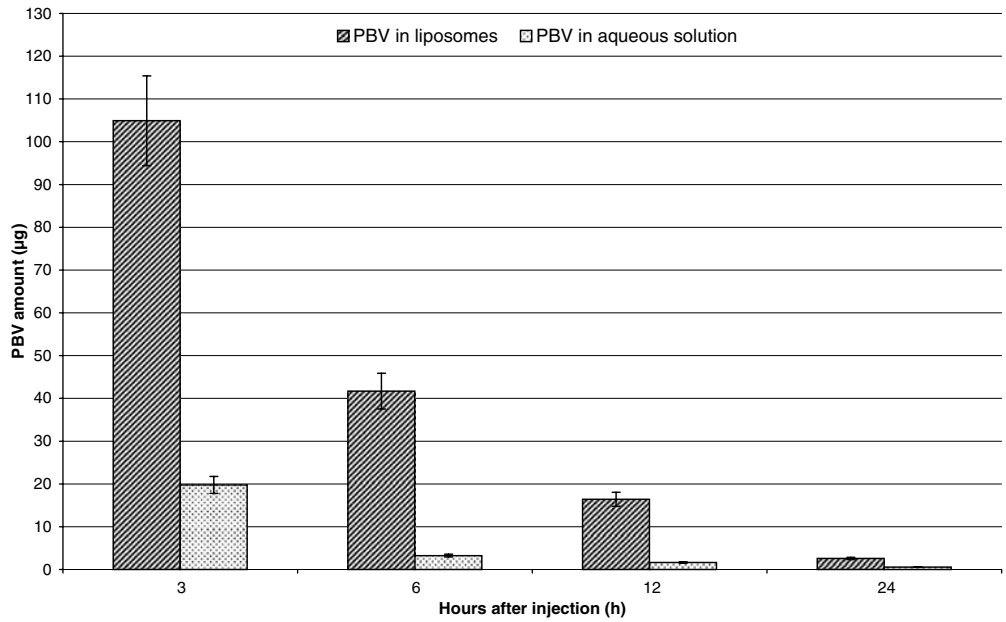

Fig. 5 Amount of Patent Blue in regional lymph nodes after injection into the mammary glands.

4. DISCUSSION

For the sentinel node procedure, it is vital to clearly stain the lymph nodes. Otherwise, it is not possible to localize the nodes and to discern them from their surrounding tissue.

Different substances tested for this purpose in the past are not used in practice due to their insufficient staining properties or their toxicity. As an example, in one study *(35)* the dark blue dye Guajazulen dissolved in Lipiodol Ultra-Fluid was used. Endolymphatically administered, this substance demonstrated excellent staining properties. Unfortunately, it caused remarkable side effects with oil droplets leading to tissue compression and necrosis. Moreover, lung involvement with subsequent respiratory distress was observed. Guajazulen dissolved in Lipiodol Ultra-Fluid was, therefore, not recommended for clinical practice.

In the following animal experiments, sufficient staining of lymph nodes could be achieved by direct endolymphatic injection of liposomes carrying blue dye *(1,36,37)*. A similar effect was observed in the clinical application of liposomal blue dye *(38)*. The presence of blue dye influenced not negatively the histological examination *(39)*.

In experiments on dogs, PBV concentrations of 100 µg/g could be achieved in lymph node tissue. Unfortunately, this technique is time consuming and requires a skilled expert. After subcutaneous administration into the hind pad of dogs, which is easy to perform, only concentrations up to 15 µg/g tissue could be reached *(36)*.

Lymph nodes, which carried metastases, were expected not to be accessible for staining. Fortunately, it was reported that there is no effect of lymphatic tumor burden on sentinel lymph node biopsy in breast cancer *(16)*.

In the current study, it was demonstrated that PBV concentrations of up to 67 µg/g tissue can be reached in lymph nodes after subcutaneous injection. This is of clinical relevance, as a clear identification of lymph nodes is possible from concentrations of 7 µg/g lymph node tissue on.

The clinical application of findings seems to be possible, as no adverse reactions were caused by the liposomes. This effect might be explained by the fact that the liposomes

consist of substances, which are physiologically present in lymph. In addition to the lack of adverse effects, liposomes seem to be preferable to other lipoidal carriers. Liposomes neither lead to embolization of blood capillaries nor are they stored in the lungs. Liposomes do not destroy lymph node parenchyma. Moreover, they do not cause allergic reactions or pain in patients.

All in all it can be concluded that liposomes are suitable for indirect lymphography and have much better staining properties as aqueous solutions. They transport much more pharmaceuticals to the lymph nodes, are nontoxic, and are not influencing the diagnostic value of the histological examination. Their clinical use for preoperative staining and the identification of sentinel lymph nodes is, therefore, advocated.

The incorporation of blue dye in liposomes resolves the known disadvantages of aqueous solution. The liposomal Patent Blue V (PBV) can probably replace radionuclide labeling in all cases, where it is of importance that the lymph nodes are visible to the naked eye and no radioactive contamination occurs.

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VI Molecular Mechanisms of Metastasis

19 The Role of Lymphangiogenesis in Regional Lymph Node Metastasis: Animal Models

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CONTENTS

ABSTRACT

A combination of clinicopathological, molecular, and sophisticated animal experiments have demonstrated the critical importance of lymphangiogenesis in the pathogenesis of sentinel lymph node (SLN) metastasis. This chapter highlights the animal models and experiments that confirmed the association of tumoral and peritumoral lymphangiogenesis and metastasis. The initiating events may have been the observation of lymphatic development in embryos. Using classic embryological models and modern molecular techniques allowed a dissection of the developing lymphatic system that previously was unattainable. Lymphangiogenesis has also been identified in wounds, inflammation, infections, and tumors. The biochemical and molecular events in embryologic development of the lymphatic system were used in animal models of tumors to identify lymphangiogenesis and to dissect the sequence of events during tumor dissemination. The tools available for these experiments have continued to advance and are summarized in the chapter.

Key Words: lymphangiogenesis; lymph node metastasis; animal models

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1. INTRODUCTION

Metastasis to the sentinel and subsequent regional lymph nodes (SLNs and RLNs) from a primary tumor is accomplished by finely integrated mechanical, genetic, and molecular processes *(1,2)*. The pace of discovery of these processes has been enhanced through a combination of clinicopathological observations *(1)* and sophisticated animal experiments. None of these discoveries would have been possible without exciting and careful biochemical and molecular studies that yielded a continually expanding knowledge of unique markers on lymphatic endothelial cells (LECs) *(3)*.

Tumor angiogenesis was observed functionally and morphologically before the discovery of vascular endothelial growth factors (VEGFs) *(4,5)* Tumor lymphangiogenesis was initially not even imagined because it was difficult to distinguish between blood vessel capillaries and lymphatics *(1)*; yet pathologists had enough intuition to speculate that tumor cells invaded lymphatics. Even today tumor cells, when seen by standard light and electron microscopy in a space lined by endothelial cells, are identified by the relatively crude, nonspecific term "angiolymphatic invasion."

Clinically, angiolymphatic invasion is associated with SLN metastasis *(6)*. We assume that the original observations of Ewing in 1925 *(7)* can be translated to mean that tumor cells invaded lymphatic capillaries and embolized to the RLNs following an orderly anatomic pathway. The mechanisms of lymphatic invasion and embolization have been partially elucidated by experimental studies in animal models.

Tumor-associated lymphangiogenesis was identified because of newly identified markers on LECs, and cloning of genes for these markers *(3,8–11)*. Lymphangiogenesis occurs during embryological development and, in the adult, during inflammation, wound healing, infections, and in association with tumor progression *(12,13)*. The most important proteins involved in lymphangiogenesis are the VEGF-C/VEGF-D/VEGFR-3 signaling system. Although VEGF-C and VEGF-D can induce angiogenesis by signaling through VEGFR-2/Flk1/KDR *(14)*, it stimulates lymphangiogenesis through VEGFR-3 *(15,16)*.

By interfering with the expression of genes for the VEGF receptor-3 (VEGFR-3), which binds VEGF-C, it was possible to recognize the lethality of nonexpression. Dissection of the origins of the lymphatic system from high endothelial venules (HEVs), especially in the neck and caudal regions of the embryo, was also studied. Other experiments showed the importance of Prospero-homeobox-1 (Prox1) expression, and also the normal expression of other receptors and signaling proteins in lymphatic embryogenesis *(13)*.

Direct visualization of abundant dilated tumor-associated lymphatics in intact animals by microlymphangiographic techniques has further confirmed the molecular and embryological studies *(17,18)*. While spontaneous metastasis in animal experiments cannot exactly mimic human tumor biology, syngeneic tumors in immunologically intact animals and xenogeneic tumors in immunologically compromised animals provide an experimental milieu that allows "pure" Newtonian–Gallilean scientific questions to be addressed. The truths and excitement of scientific discovery assure us that many details still remain unanswered, and it has only very recently been shown that tumor-associated lymphangiogenesis is not only found in and around the primary tumor. SLN lymphangiogenesis occurs even before tumor cells are identified in the node *(18–20)*. These observations will be discussed later in the chapter, but it is valuable to recognize that such observations in animals have been confirmed in human tumors and the important biological insights that are accrued by these means will hopefully translate into helpful new clinical treatment modalities.

This chapter is devoted to animal models of lymphangiogenesis and to other models in which the relationship between lymphangiogenesis and RLN metastasis has been proven.

2. ANIMAL MODELS OF LYMPHANGIOGENESIS

2.1. Embryos

Embryonic lymphangiogenesis has been extensively studied in the developing mouse *(13)*, where lymph vessels began to appear around embryonic day 10 (E10). Prox1 and VEGF-C were vital and essential for the initial sprouting of LECs from jugular and perimesonephric veins. Migration of the primitive LECs developed into primary lymph sacs and a lymphatic plexus. Prox1 deletion was lethal in the perinatal period. VEGF-$C^{-/-}$ mouse embryos lacked a lymphatic vasculature. These embryos could be rescued by exogenous administration of VEGF-D protein. However, VEGF-$D^{-/-}$ embryos showed normal lymphatic vascular development. VEGFR-3 deletion resulted in mid-gestation death with major abnormalities in blood vessel development. Lymphatic vessel hyaluronan receptor-1 (LYVE-1) was first identified on E9 but deletion of the receptor did not affect lymphatic vessel development.

Separation of blood and lymphatic vessels was highly dependent on SyK and SLP-76. Other ligands and receptors, such as angiopoietins, Tie receptors, Ephrin B2, Foxc2, podoplanin, Nrp2, Erk3, Pik3r1, and Sox18, have been studied and the effects of knockdown or knockout (KO) genes on developing embryologic lymphatic vessels were reported *(3,10,11,21,22)*. Differential expression of LEC molecular markers at various stages of embryologic development may perhaps be mimicked in the lymphangiogenesis associated with tumors.

2.2. Skin Injury Models

Some of the most frequently used and the most established animal models to study lymphangiogenesis are skin injury models. These models have been developed in mice, rats, rabbits, and pigs.

The earliest studies in lymphangiogenesis were undertaken by Clark and Clark *(23)*, utilizing the model of transparent chambers in rabbit ears, demonstrating outgrowth of lymphatic capillaries from existing vessels. Additional studies over the years with the rabbit ear model demonstrated a time course of newly formed lymphatics *(24)*.

In a pig wound-healing model, new VEGFR-3 positive lymphatic vessels appeared at about the same time as blood vessels, but regressed earlier *(25)*. Expression of VEGF-C from an adenoviral vector induced lymphatic vessel production in the skin of mice, as well as upregulated VEGFR-3 *(26)*. Expression of a spliced variant of VEGF-D, Ad-VEGF-DNC, in the skin of rats led to the development of slit-like structures which stained positive for VEGFR-3 *(27)*.

2.3. Avian Chorioallantoic Membrane

A popular model in which to study lymphangiogenesis is the avian chorioallantoic membrane (CAM) model. Although CAMs have been used as a model for understanding angiogenic factors since 1974 *(28)*, the use of CAM to study lymphangiogenesis was first described in 1997 *(29)*. Oh and colleagues demonstrated that VEGFR-3 was found only on lymphatic endothelial cells (LECs), whereas VEGFR-2 and VEGR-3 were found on both BECs and LECs. Furthermore, the authors demonstrated that neither various isoforms of VEGF nor placental growth factor (PLGF-1 or PLGF-2) had an effect on lymphangiogenesis, but that VEGF-C induced the growth of many lymphatic vessels. As only VEGFR-3 is expressed on the lymphatic vessels adjacent to the blood vessels, the authors concluded that VEGF-C signaling through binding its receptor VEGFR-3 on these endothelial cells leads to lymphangiogenesis.

2.4. Mouse Cornea

Chang and colleagues *(30)* used a modified murine cornea neovascularization model to induce lymphangiogenesis. The usual dose of βFGF-induced ingrowth of new blood vessels into the cornea. When they inserted a corneal pellet containing a low concentration of βFGF lymphatic vessels, but not blood vessels, grew from the limbus to reach the implanted pellets. This development of new lymphatic vessels by βFGF was mediated by VEGF-C and VEGF-D through VEGFR-3 signaling, demonstrated through the use of neutralizing antibodies to VEGFR-3 *(31)*. Use of Angiopoeitin-1-containing pellets inserted into murine corneas demonstrated the generation of new lymphatics that was blocked through the administration of Tie-2-blocking antibodies *(32)*. Using the murine model of inflammatory corneal damage, Dietrich et al. demonstrated that lymphangiogenesis also requires integrin V *(33)*. This is most likely due to the need for integrin V for VEGFR-3 signaling *(34)*.

2.5. Mouse Tail

A newer model of lymphangiogenesis was created by removing a 2-mm wide circumferential ring of skin, subcutaneous tissue, and lymphatics from the tail of an adult mouse. The bone and blood vessels were left intact. The vacant space was replaced with a fitted gas-permeable silicone collagen-filled sleeve *(35)*. In this model, the growth of new lymphatic vessels was visualized across the collagen-filled sleeve. This model has been used to demonstrate the time frame of lymphangiogenesis correlated with the expression of various proteins involved, such as VEGF-C *(36)* and matrix metalloproteases. The distal tail developed an increase in interstitial fluid and the resultant increase in interstitial fluid pressure (IFP), together with increased VEGF-C secretion at the cut edge, and the expression of matrix metalloproteases, connected the cut ends with fluid-filled spaces which were eventually lined by new LECs lining the spaces *(37)* (see Fig. 1). Combining this model with neutralizing antibodies to either VEGFR-2 or VEGFR-3 at select times demonstrated that both VEGFR-2 and VEGFR-3 were required for the migration and proliferation of lymphatic vessels, but that VEGFR-3 was not required for organization *(38)*.

2.6. Regenerating Lizard Tail

New animal models continue to be developed to study the timing and genetics of lymphangiogenesis. One such model is the regenerating lizard tail model *(39,40)*. In this model, the lizard tail was shed through autotomy, and the new tail regenerated with a completely new functional lymphatic system. This system showed that increased IFP was not always necessary to stimulate new lymphatic vessel development. Lizard homologues of VEGF-C/VEGF-D were also found to be upregulated and important to lymphangiogenesis within the newly formed tail *(39)*. Furthermore, the lizard tail model allowed for study of the timing of new lymphatic growth *(40)*.

2.7. Tadpoles

Genetic manipulation of the developing Xenopus tadpole has demonstrated the requirement of Prox1 for lymphangiogenic but not angiogenic development *(41)*, similar to the Prox1-null mouse *(42)*. Furthermore, the function of VEGF-C and VEGF-D was similar amongst the tadpoles and the knockdown mice. This model, therefore, offers a much more manageable system in which to study the genetic factors involved in lymphangiogenesis.

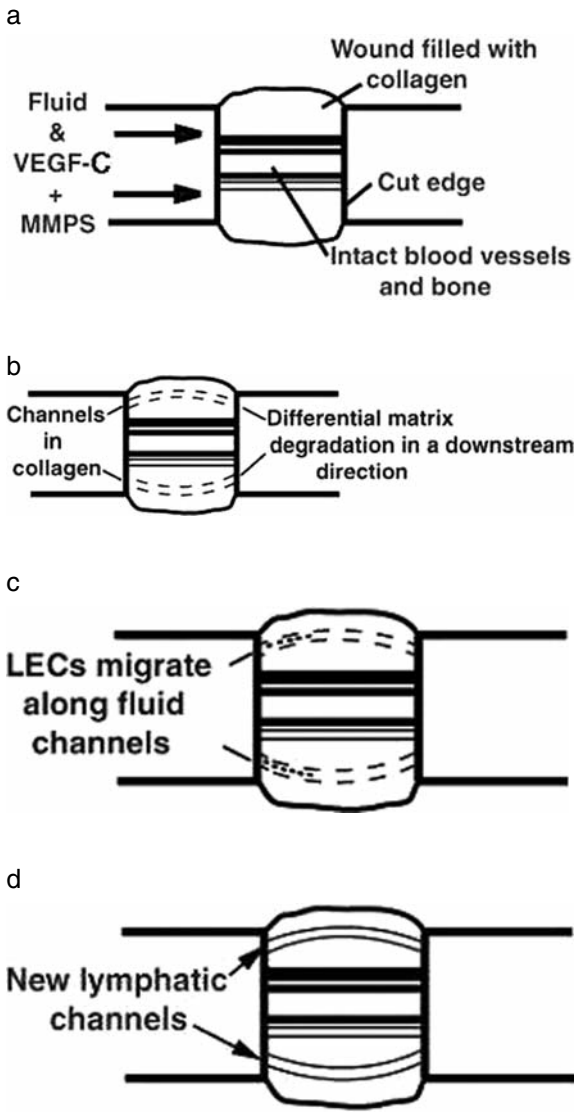

Fig. 1 (a) Mouse tail lymphangiogenesis model derived from Boardman et al. (2003) *(35)*. (b) Development of spaces in collagen through matrix degradation. (c) Migration of LECs into fluid spaces. (d) New lymphatic channels.

2.8. Zebrafish

Recently, a lymphatic system that is sensitive to VEGF-C and VEGFR-3 alterations was described in Zebrafish, adding an additional model in which to study the development of the lymphatic system in a much shorter time frame than most of the other models described *(43)*.

2.9. Postnodal Lymphatic Vessels in Sheep

A new model was developed in order to more clearly visualize the physiological properties of newly formed lymphatics, utilizing the obstruction of large postnodal lymphatic vessels in sheep *(44)*. This model again reaffirmed the role of Prox1, VEGF-C/ VEGF-D, and Tie-2 in the regeneration of lymphatic vessels.

2.10. Transgenic Mouse Models

The development of transgenic mouse technology has significantly advanced the understanding of the roles of specific genes in lymphangiogenesis.

Knockout of VEGF-C is lethal, demonstrating that it is essential for the formation of lymphatic vessel sprouts via paracrine signaling *(45)*.

Overexpression of VEGF-C in transgenic mice induced a proliferation of specific LECs with hyperplasia of the lymphatic vasculature *(46)*. Furthermore, overexpression of VEGF-C under the rat insulin promoter (Rip2-VEGF-C) led to the development of significant lymphangiogenesis surrounding the islets of Langerhans *(47)*. Rip2-VEGF-C mice were similar in phenotype to angiopoietin-2 (Ang-2)-deficient mice. Mice lacking Ang-2 lacked functional lymphatic structures as well as vascular abnormalities. However, the lymphatic deficits were rescued by Angiopoietin-1 (Ang-1), thus demonstrating that Ang-2 normally functions as an agonist to Tie-2 *(48)*. Ang-1 and Ang-2 signal through the Tie-2 receptor tyrosine kinase. A direct interaction between Tie-2- and VEGFR-3-signaling pathways has not been demonstrated, although Ang-1 does induce an increase in VEGFR-3 signaling and increases lymphatic cell proliferation, as well as lymphatic sprouting *(11)*.

The development of the lymphatic structures within the dermis of transgenic mice was blocked by the expression of a soluble VEGFR-3 under a keratin-14 promoter, demonstrating that signaling via VEGF-C and VEGF-D was through VEGFR-3 *(49)*. This work also demonstrated that continuous signaling throughout development of lymphatic vessels was necessary, as regression of existing fetal lymphatic vessels was seen. Overexpression of VEGF-D and a specific VEGFR-3-signaling mutant of VEGF-C (VEGF-C156S) under the same keratin promoter in transgenic mice demonstrated that signaling through VEGFR-3 via VEGF-D and VEGF-C was sufficient to produce lymphangiogenesis.

Prox1 was required not only for budding and sprouting of lymphatic vessels but also for differentiation of endothelial cells into lymphatic phenotype *(42)*. Expression of Prox1 was an initiating event in the budding of LECs from venous endothelial cells during development *(42,50)*. Prox1 was also important for the directionality of the budding, thus suggesting that Prox1 is part of a yet uncharacterized maintenance-signaling pathway.

Neuropilin-$2^{-/-}$ ($Nrp2^{-/-}$) mice lacked small lymphatic vessels, with normal development of larger lymphatic vessels *(51)*. Thus, Npr2 was required for the formation and location of small lymphatic vessels.

T1/podoplanin$^{-/-}$ mice, while developing a peripheral lymphatic system, were characterized by defects in patterning and later stages of development *(52)*.

Lyve-1 is a LEC marker, but the function of Lyve-1 is not clearly delineated. Lyve-$1^{-/-}$ mice have increased lymphatic flow, thus suggesting that Lyve-1 may mediate signaling involved in controlling flow rates *(53)*.

Other genes involved in lymphangiogenesis include *Syk*, *SLP-76*, *PLC*, as well as *Spred-1* and *Spred-2*. Syk and SLP-76 hematopoietic signaling proteins are key to the separation of lymphatic vessels from blood vessels *(54)*. In $Syk^{-/-}$ as well as SLP-$76^{-/-}$ mice, there was a mixing of the blood and lymph within the lymph tissue, demonstrating that a clear separation of the two did not occur *(54)*. Furthermore, transplantation of SLP-$76^{-/-}$ bone marrow into lethally irradiated mice led to a lack of development of proper lymphatic system within the mesentery. Syk and SLP-76 signaled in concert with PLC by binding immunoreceptor tyrosine-based activation motif (ITAM)-bearing receptors as well as integrins. However, linker for T-cell activation $(LAT)^{-/-}$ mice do not emulate the same pathogenesis of $Syk^{-/-}$, SLP-$76^{-/-}$, and $PLC^{-/-}$ mice *(55)*. Thus, the signaling pathways involved in the separation of lymphatic vessels from blood vessels need further delineation.

Spred-1 and Spred-2 have also been implicated in lymphangiogenesis, as Spred-1/2 KO mice have a similar phenotype in regard to lymphatic vessel separation, to the $Syk^{-/-}$, $SLP\text{-}76^{-/-}$, and $PLC^{-/-}$ mice *(56)*. Interestingly, while Syk, SLP-76, and PLC appear to be downstream modulators of VEFGR3 signaling, Spred-1 and Spred-2 appear to be negative regulators. Additionally, integrin $ix^{-/-}$ mice suggest a requirement for this integrin subunit; although no specific structural abnormalities could be found, there was a lack of the development of the thoracic duct and other lymphatic leakage abnormalities *(57)*.

The lymphotoxin-signaling (LT-R) pathway was demonstrated to be important in inflammatory lymphangiogenesis within the thyroid *(58)*. The TGCCL21 transgenic mouse model, which overexpressed the chemokine CCL21 within the thyroid *(59)*, was used to demonstrate that lymphatic vascularization occurred within the thymus of these animals. This contribution to lymphangiogenesis was shown to be dependent on signaling through the LT-R pathway by crossing the $TGCCL21^{-/-}$ mice to LT-BR-transgenic mice.

VEGF-C mRNA steady-state levels were increased in a time- and concentration-dependent manner by interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and IL-1α in human lung fibroblasts *(60)*. In contrast, IL-1β appeared to downregulate Ang-1 mRNA, and this effect was inhibited by Dexamethasone and IL-1 receptor antagonist. This raises the possibility that certain proinflammatory cytokines can regulate lymphangiogenesis indirectly via VEGF-C.

2.11. Peri- and/or Intratumoral Lymphangiogenesis

The once prevalent belief that tumor cells metastasize strictly via preexisting lymphatic channels has recently given way to the notion that primary tumors induce new lymphatic channels by secretion of lymphangiogenic cytokines. VEGF-C secreted by tumor cells induced LEC chemotaxis (causing LECs to migrate toward VEGF-C-producing cells), but not chemoinvasion (i.e., proteolytically dependent LEC invasion through an extracellular matrix). Other factors were required for fluid channel formation and subsequent invasion, including proteases and growth factors secreted by the tumor cells *(61)*. In the process of tumor lymphangiogenesis, LECs sent long filopodia toward the VEGF-C-producing tumor cells and formed tumor-directed vascular sprouts *(13)*. LEC migration occurred in the direction of interstitial flow and lymph flow *(35)*.

The most important of these modulators appear to be members of the VEGF family, including VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D, VEGF-E, and PLGF, all key regulators of physiological and pathological vasculogenesis, hematopoiesis, angiogenesis, lymphangiogenesis, and vascular permeability.

VEGF-C, originally isolated in 1996 from human prostatic carcinoma cells, was the first selective lymphangiogenic factor identified *(9)*. This growth factor induced lymphangiogenesis without accompanying angiogenesis through its interactions with the transmembrane tyrosine kinase receptor VEGFR-3 *(29)*. Unlike VEGFR-2, which was found primarily on BECs, VEGFR-3 was predominantly expressed in LECs *(62)*. VEGFR-3 has been shown to play a major role in embryological angiogenesis, but is later downregulated in BECs during development, and is found almost exclusively in LECs and fenestrated blood vessel endothelium postnatally *(63)*. Indeed, continuous VEGFR-3 signaling is essential for proper fetal development and maintenance of the lymphatic vascular system, and anti-VEGFR-3 antibodies have been shown to induce regression of previously formed lymphatics secondary to LEC apoptosis *(49)*.

VEGF-C has been shown to promote both lymphangiogenesis and angiogenesis in melanomas, but only lymphangiogenesis in breast cancer models. These differing roles may be related to in vivo

proteolytic processing of pro-VEGF-C, as the 21-kDa form predominates in melanomas, while breast cancer tumors express mostly the 31-kDa form, which selectively activated VEGFR-3 *(63,64)*. This angiogenic ability is most likely mediated by VEGFR-2, although VEGF-C preferentially stimulates VEGFR-3 autophosphorylation over VEGFR-2 *(63,65)*. Interestingly, recent evidence has shown that heterodimer formation of VEGFR-3 with VEGFR-2 might alter VEGFR-3 phosphorylation site usage in LECs, thereby adding another level of complexity to VEGF-C modulation of VEGFR-3 signaling *(66)*.

VEGF-D (c-fos-induced growth factor, FIGF) is the member of the VEGF family most closely related to VEGF-C, by virtue of unique N- and C-terminal extensions not found in any other VEGF growth factors *(67)*. In fact, VEGF-C and VEGF-D are the two best-known lymphangiogenesis factors and the only known ligands for VEGFR-3 *(18)*. Like VEGF-C, the mature form of VEGF-D bound to and activated both VEGFR-2 and VEGFR-3 was mitogenic for ECs and was both angiogenic and lymphangiogenic in vivo *(68)*. But VEGF-D differed from VEGF-C in several important respects. While VEGF-D induced lymphangiogenesis and promoted metastasis to SLNs and distant sites, it has been shown to repress hemangiogenesis and tumor outgrowth in a pancreatic β-cell cancer model in mice *(62,69,70)*. VEGF-C, on the other hand, induced lymphangiogenesis, angiogenesis, and nodal metastasis without provoking distant metastasis or suppressing angiogenesis in this model *(62)*.

VEGF (VEGF-A) has been identified as the predominant tumor angiogenesis factor in the majority of human and experimental murine cancers, acting via the VEGF-1 and VEGF-2 receptors *(5)*. VEGF-A has also been shown to play a role in lymphangiogenesis using a transgenic mouse model overexpressing VEGF-A *(18)*. In this model, VEGF-A induced active proliferation of VEGFR-2-expressing tumor-associated lymphatic vessels as well as tumor metastasis to the sentinel and distal lymph nodes.

Hepatocyte growth factor (HGF), secreted by tumor cells, was able to stimulate peritumoral lymphatic growth via VEGFR-3-mediated signaling pathway *(71)*. However, results have been inconsistent in regard to the VEGFR-3 pathway. Cao et al. *(71)* found that HGF-induced lymphangiogenesis was partially blocked by soluble VEGFR-3, while Kajiya et al. *(72)* found that anti-VEGFR-3 antibody was unable to block the HGF effect, indicating that HGF may also directly promote lymphangiogenesis by an alternate mechanism.

Platelet-derived growth factor-BB (PDGF-BB), which can activate the *Akt* kinase for promoting antiapoptotic signals, has been implicated to be a pleiotropically controlling survival factor as potent as VEGF-C in vivo in inducing intratumoral lymphangiogenesis without mediation via the VEGF-C/VEGF-D/VEGFR-3 pathway in a mouse fibrosarcoma model. The lymphatic formation induced by PDGF-BB and a maximal response for further remodeling occurred at days 5 and 14 after tumor implantation, respectively. Similar to tumor angiogenesis, these premature tumor lymphatics seemed to be leaky and resulted from fusion of initial lymphatics to large lumens *(73)*.

Src Kinase has been shown to interact with insulin-like growth factor-1 (IGF-1), and in a recent study, Tang et al. *(74)* showed that activation of the IGF-1 receptor increased VEGF-C expression through the phosphatidylinositol-3-kinase-dependent pathway *(75)*. Src has also been reported as an upstream receptor of p38 in sphingosine-1-phosphate-mediated angiogenesis, with p38 known as a key mediator of VEGF-C upregulation in breast and lung cancer *(76,77)*.

A number of small-molecule kinase inhibitors of VEGFR-2 have been found to also inhibit VEGFR-3 signal transduction, including SU11248, AZD2171, Bay 43-9006, PTK/ZK, MAZ51, and CEP-7055. These small molecular compounds also affect a number of other tyrosine kinases, and their distinct effects on tumor lymphangiogenesis in vivo remain to be investigated *(78–83)*.

3. ANIMAL MODELS THAT DEMONSTRATED THE RELATIONSHIP BETWEEN LYMPHANGIOGENESIS AND LYMPH NODE METASTASIS

3.1. Lymphangiogenesis Associated with the Primary Tumor

The role of lymphangiogenesis in RLN metastases has recently become the subject of intense scrutiny, as several modulators of lymphangiogenesis have been shown to play integral roles in regional tumor metastasis. SLN metastasis has been found to be directly correlated with the development of lymphangiogenesis in many different animal models (Table 1). For example, Mandriota et al. *(47)* found that the overexpression of VEGF-C in pancreatic islet cell tumors of transgenic mice induced lymphangiogenesis and promoted lymph node metastasis. In this model, VEGF-C production was regulated by the Rip promoter, which is known to be transiently active in neural tube and neural crest cells during embryogenesis. Rip-Tag2, a well-studied transgenic murine model of pancreatic β-cell carcinogenesis, formed tumor cell aggregates within preexisting lymphatics but did not go on to develop lymph node metastases. However, Rip-VEGF-C × Rip-Tag2 double-transgenic tumors successfully formed lymph node metastases in 37% of cases, and the incidence of tumors formed more than doubled.

Neutralization of VEGF-C and VEGF-D in an experimental tumor model by systemic overexpression of a soluble VEGFR-3 fused to the Fc domain of immunoglobulin γ chain (VEGFR-3-Ig) inhibited lymphangiogenesis and lymph node metastasis *(84)*. This occurred by "trapping" the available VEGF-C and VEGF-D with decoy receptors before they could interact with VEGFR-3.

VEGFR-3-Ig (anti-VEGFR-3 antibody) suppressed lymphangiogenesis and lymph node metastasis, but did not prevent lung metastasis, implying that the mechanisms for lymphatic and lung metastasis may differ *(84)*. This may be because suppression of VEGFR-3 activation prevents lymphatic metastasis, but does not affect systemic metastasis. VEGF-C overexpression and subsequent de novo lymphatic vessel formation were necessary, but not necessarily sufficient, for metastatic dissemination of tumor cells to the lymph nodes. Additional factors were clearly needed in order for metastasis to occur.

Table 1
Animal Models in Which Sentinel Node Metastasis was Directly Correlated with the Development of Lymphangiogenesis

Animal model	*Tumor type*	*Lymphangiogenic cytokine*	*References*
Nude mice	A375 Melanoma	VEGF-C	*(93)*
SCID mice	EBNA	VEGF-D	*(70)*
Nude mice	Breast	VEGF-C	*(94,95)*
Transgenic	Pancreas β-cell	VEGF-C	*(47)*
SCID mice	Breast	VEGF-C	*(76,96)*
Nude mice	Fibrosarcoma	VEGF-C	*(97)*
C57 BL/6	Melanoma	VEGF-C	*(97)*
Nude	Breast	VEGF-C	*(98)*
Wistar rat	Breast	VEGF-C	*(99)*
Transgenic	Pancreas β-cell	VEGF-C	*(100)*
C57 BL/6	Fibrosarcoma	PDGF-BB	*(73)*
SCID mice	Lung	VEGF-C	*(17)*
Transgenic	Squamous	VEGF-A	*(18)*
C57 BL/6	Fibrosarcoma	VEGF-A	*(101,102)*
Nude	Rectal	VEGF-C	*(88)*

Other studies have shown that inhibition of VEGFR-3 activation with an antagonistic antibody suppressed LNM more potently than inactivation of VEGFR-2, although treatment with both anti-VEGFR-2 and anti-VEGFR-3 antibodies more potently decreased lymph node metastasis (LNM) and distant metastases than either antibody alone *(49,85)*.

Anti-VEGFR-3 antibody reduced both lymphatic hyperplasia and the delivery of tumor cells to the draining lymph node, and led to a reduction in lymph node metastasis by inhibition of the lymphangiogenic VEGF-C effect. However, this treatment was unable to prevent the growth or metastasis of tumor cells already seeded in lymph nodes prior to administration of anti-VEGFR-3 antibody. Thus, therapies directed against VEGF-C and/or VEGFR-3 appeared to target the earliest steps of lymphatic metastasis *(17,84,86)*. Blocking of VEGFR-3 signaling inhibited the entry of tumor cells into the lymphatic vessels and decreased their transit into the lymph nodes, but had little effect on later stages of tumor metastasis *(17)*.

3.2. Lymphangiogenesis Associated with the Sentinel Lymph Node

Qian et al. *(20)* showed that the primary tumor induced a number of structural changes within the microenvironment conducive to metastasis, marked by vascular reorganization within the SLN. The SLN was stimulated to become a functional blood and lymphatic vessel-enriched organ before and independent of metastasis**.** The total cross-sectional area of lymph vessels/sinuses in the SLN was highly correlated with the primary tumor weight, and mean wall thickness in the presence of a primary tumor was significantly less than in the noncancerous control group. This appeared to be due to lymphatic dilatation, with normally thick-walled HEVs converted into flat endothelial cells, which gradually lost the HEV marker MECA-79 from the tumor margin to the central part of the metastatic tumor nest. The use of athymic nude mice in this study supports the theory that this effect was due to the influence of the primary tumor, rather than an immune response to micrometastases or tumor antigens from the primary lesion. The SLN was reorganized by the primary tumor to become a functional blood vessel-enriched organ before and independent of metastasis. The cellular morphology of the tall endothelial cells forming normally thick-walled HEVs changed dramatically to become flat endothelial cells in more dilated vessels in the presence of a primary tumor. These changes were accompanied by a gradual loss of the HEV marker MECA-79 from the tumor margin to the central part of the metastatic tumor nest.

Harrell et al. *(19)* showed in the syngeneic C57BL/6 murine melanoma model that injection of tumor cells induced lymph node lymphangiogenesis even before the tumor cells reached RLNs, indicating that the primary tumor induced these alterations from a distance. In this way, the primary tumor "prepared a bed" for tumor cells embolizing from the primary tumor via VEGF-C- and VEGF-D-induced lymphangiogenesis (Fig. 2). B-lymphocytes were also apparently required for lymphangiogenesis and enhanced lymphatic flow through the lymph node to occur, as these changes were not observed in B-cell-deficient mice. Interestingly, B cells and T cells were shown to accumulate in the tumor-draining lymph node, but were not appreciably increased within the primary tumor itself.

Hirakawa et al. *(87)* developed transgenic mice that overexpressed VEGF-C and green fluorescent protein in the skin. They studied chemically induced tumors in the skin. In contrast to VEGF-A, VEGF-C did not increase the growth of primary tumors. Instead the SLNs demonstrated an extensive expansion of lymphatic networks, even before the onset of metastasis. SLN lymphangiogenesis increased upon arrival of tumor cells in the node. This process also increased the likelihood of metastasis to more distant nodes and to systemic sites.

Additional studies have confirmed that the traditionally "lymphangiogenic" members of the VEGF family, VEGF-C and VEGF-D, induced SLN lymphangiogenesis, thereby increasing the likelihood of metastasis to SLNs as well as more distant nodes and systemic sites

Fig. 2. A cartoon that demonstrates "preparation of the bed"—in this example, VEGF-C- and VEGF-D-stimulating sentinel node lymphangiogenesis before tumor cells appear in the sentinel lymph node (SLN).

(47,62,70,84,87,88). Some recent studies have unexpectedly shown that VEGF (VEGF-A), a traditionally "angiogenic" member of the VEGF family, may play a role in SLN lymphangiogenesis. Although the mechanism for this lymphangiogenic VEGF-A effect remains unclear, current theories include recruitment of VEGF-C/VEGF-D-secreting macrophages *(89)*, and interaction with the VEGFR-2 *(18)* and/or VEGFR-3 *(90)* receptors. As with VEGF-C and VEGF-D, this VEGF-A effect appears even before metastasis has occurred, and there is mounting evidence that both VEGF-A and VEGF-C may be essential for SLN lymphangiogenesis *(91)*.

4. SUMMARY

Multiple steps are required for tumor cells to metastasize from their primary site to RLNs. These steps include detachment from the primary tumor mass, invasion into lymphatic vessels, transport through draining lymphatic vessels, arrest in lymph nodes, and survival and growth in lymph nodes *(92)*.

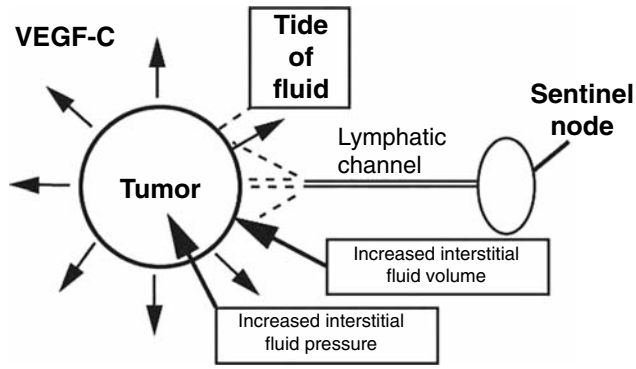

Fig. 3. Mechanical, enzymatic, and molecular integration results in peritumoral lymphangiogenesis. Lymphangiogenesis is necessary for metastasis to the SLN in most of the models studied.

Animal models have demonstrated peri- and intratumoral lymphangiogenesis and, recently, lymphangiogenesis in the draining sentinel node. Figure 3 diagrams the interplay between the best-studied lymphangiogenic cytokine, VEGF-C, and tumoral fluid dynamics and matrix metalloproteases. A deeper understanding of the importance of lymphangiogenesis and the likelihood of SLN metastasis is likely to yield potentially important therapeutic information. Human and animal clinical cancer management advances, brought from the animal laboratory to the bedside, will enable us to better treat our patients.

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20 Sentinel Lymph Node Chemokine Microenvironment Modulated by Melanoma Metastasis

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CONTENTS

ABSTRACT

Primary melanoma tumor-draining lymph nodes (LNs) play a significant role in controlling regional metastasis. Chemokine receptors CXCR4 and CCR7 are expressed on immune cells, whereby their respective ligands are, CXCL12 and CCL21. We hypothesized that melanoma metastasis to the sentinel LN (SLN) suppresses chemokine production, creating a more favorable tumor microenvironment for metastasis. A quantitative real-time reverse transcription PCR (qRT) assay was used to assess CXCL12 and CCL21 expression in paraffin-embedded (PE) SLN from melanoma patients ($n = 124$). SLN metastases were diagnosed by hematoxylin and eosin (H&E) and immunohistochemistry (IHC), and classified as macrometastases (>2.0 mm) or micrometastases (≤2.0 mm). CXCL12 and CCL21 levels were significantly enhanced in SLN with micrometastases than in SLN with macrometastases (CXCL12, $p = 0.02$; CCL21, $p = 0.006$) or in SLN without metastasis (CXCL12, $p = 0.04$; CCL21, $p < 0.001$). IHC analysis of SLN showed that the chemokine mRNA expression correlated with protein expression. Increase in primary tumor burden significantly correlated with suppressed CXCL12 ($p < 0.0001$) and CCL21 ($p < 0.05$) expression in the SLN. Progression of nodal metastasis burden and primary tumor burden was shown to significantly suppress

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both SLN CXCL12 and CCL21. Reversal of this chemokine suppression may improve the immunity of draining LN against tumor invasion.

Key Words: chemokines; sentinel node; melanoma; CCL21; CXCL12

1. INTRODUCTION

Lymph node (LN) metastasis is an important prognostic factor influencing disease outcome in cutaneous melanoma *(1)*. Identified by lymphatic mapping, the sentinel lymph node (SLN) represents the first regional LN to which a primary tumor drains and metastasizes *(2,3)*. Tumor-draining LNs are activated during melanoma development; however, failure to control primary tumor progression eventually results in metastasis to the regional nodal basin *(4)*. The regional tumor-draining LNs play a significant role in controlling tumor metastasis. There is significant evidence that immune cells bearing chemokine receptors, such as antigen-presenting dendritic cells (DCs), Langerhans cells (LCs), T cells, and natural killer (NK) cells, migrate from peripheral tissues to regional draining LNs in response to specific chemokines *(5–13)*. Chemokines are known to orchestrate immune activity in secondary lymphoid organs in response to insult that includes injury, infection, and tumor growth in the drainage region. It has also been implicated that chemokines are responsible for the recruitment of metastatic tumor cells to LNs bearing respective chemokine receptors *(14,15)*.

Chemokines belong to the chemoattractive cytokine family and are categorized into C, CC, CXC, and CX3C groups based on four conserved cysteine residues *(16)*. These chemoattractive molecules mediate their effects on target cells by interacting with G protein-linked receptors. The C-X-C ligand 12 (CXCL12; SDF-1, stromal cell-derived factor-1) and its receptor, C-X-C receptor 4 (CXCR4), are particularly relevant to cancer metastasis, as well as the immune surveillance and the homing of lymphocytes to sites of inflammation *(15,17)*. CXCL12 is highly expressed in many tissues, including lung, liver, bone marrow, and LNs, whereby stromal cells in LNs are the primary source of CXCL12 production.

CCL21 (chemokine C-C motif ligand 21) is involved in recruiting CCR7(+) naïve T cells, NK cells, memory T cells, and mature DCs *(5–10,13)* to LNs. CCL21 is constitutively expressed in high endothelial venules (HEVs) of LNs, Peyer's patches, spleen, and mucosal tissue *(8,18)*. The release of CCL21 by HEVs facilitates the recruitment of CCR7(+) cells to LNs. Because of their regulatory effects on cell recruitment to specific sites, intense scrutiny has been placed on both the CXCL12–CXCR4 and CCL21–CCR7 axes. To date, the majority of studies have focused on the role of chemokines related to immune responses, but not in response to primary tumor growth or metastasis in secondary lymphoid organs. In this study, we assessed the expression of chemokines CXCL12 and CCL21 in relation to primary melanoma and SLN metastasis burden.

The SLN is the most likely site for the earliest stages of melanoma regional LN metastasis *(2,3)* and it is the SLN that is most significantly influenced by tumor-derived factors released by primary melanoma cells. Also, the SLN is the first LN to account for the initial immune response of the regional LNs to the primary tumor *(4)*. Thus far, most studies have focused on cytokine responses in primary melanoma-draining LNs *(4)*. However, chemokine expression may also play a significant role in how effectively the LN immune system will respond to tumor progression.

We first hypothesized that early stage of metastasis to the SLN would activate chemokine expression to attract host immune cells. Second, we hypothesized that the subsequent primary tumor progression and metastatic tumor burden in the SLN would suppress chemokine production so as to dampen SLN recruitment of the immune cells facilitating the development of nodal metastasis.

2. RESULTS

2.1. CXCL12 mRNA Expression in SLN

Chemokine expression was assessed in 129 Paraffin-embedded (PE) SLN from 124 melanoma patients using an established optimal quantitative real-time reverse-transcription PCR (qRT) assay. (PE) SLN tissues from patients treated by the John Wayne Cancer Institute (JWCI) were obtained from the Division of Surgical Pathology, Saint John's Health Center. SLNs were obtained after lymphatic mapping and SLN biopsies were performed to stage-localized melanoma, as previously described *(2,3)*. All SLNs were assessed by conventional H&E and IHC staining to determine pathology status and stage of disease. SLNs were classified as node negative or node positive with micrometastases ($\leq$2.0 mm) or macrometastases (>2.0 mm) *(19)*. Of these, 92 (71%) SLNs were negative (–) for metastasis and 37 (29%) were positive (+) for metastasis by hematoxylin and eosin/immunohistochemistry (H&E/IHC) (Table 1). The 37 metastases (+) SLN stage III specimens were further categorized as either micrometastases (metastasis diameter $\leq$2.0 mm; $n = 20$) or macrometastases (metastasis diameter >2.0 mm; $n = 17$). Macrometastasis ranged from >2.0 to 15 mm with a mean of 5 mm.

PE tissue blocks were cut into 10 sections of 10-μm thickness using a new disposable sterile microtome blade for each block. Sections were deparaffinized with xylene and then digested with proteinase K. A modification of the Paraffin Block RNA Isolation Kit procedure (Ambion,

Table 1
Characteristics of Malignant Melanoma Patients

Characteristics	*Patients*
Patients	124
Sex	
Male	64
Female	60
Age	
Mean	59.0
Median	59.0
Range	13–89
SLN Specimens	129
Basin Site	
Head and Neck	36
Axilla	54
Groin	37
Other	2
TNM	
Stage I	37
Stage II	55
Stage III	37
SLN	
Metastasis(–)	92
Metastasis(+)	37
Micrometastasis	20
Macrometastasis	17

Austin, TX) was used. The RNA was quantified and assessed for purity using UV spectrophotometry and the RIBOGreen detection assay (Molecular Probes, Eugene, OR). The expression of mRNA for GAPDH, an internal reference housekeeping gene, was assessed by real-time PCR (qRT) to verify quantity and integrity of RNA in samples.

Reverse(-) transcription reactions were performed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and oligo-dT primers and random hexamers, as previously described *(20)*. The qRT assay was performed using the iCycler iQ RealTime Thermocycler (Bio-Rad Laboratories, Hercules, CA). cDNA from 250 ng of total RNA was used for each reaction *(20)*. The PCR mixture consisted of 0.2 μM of each primer, 0.5 μM FRET probe, 1 U of Ampli*Taq* Gold polymerase (Applied Biosystems, Branchburg, NJ), dNTP and buffer to a final volume of 25 μl. Samples were amplified with a precycling hold at 95° for 10 min, followed by 45 cycles of denaturation at 95° for 1 min, annealing at 60° for 1 min for CXCL12, annealing at 58° for CCL21, annealing at 55° for GAPDH, and extension at 72° for 1 min. Plasmids for individual gene cDNA were constructed as described previously *(20)*. A standard curve was generated by using threshold cycles (Ct) of nine serially diluted (10–10^8 copies) plasmids containing CXCL12, CCL21, and GAPDH cDNA for respective assays.

CXCL12 expression was reported as a ratio of CXCL12/GAPDH mRNA copy number for comparing different patients' specimens. All specimens used in the study had sufficient mRNA quantity and verified for quality of mRNA. Study analysis was performed on archival PE SLN specimens <5 years old. The mRNA copy ratio of CXCL12/GAPDH varied from 0 to 0.759 (mean ± SEM, 0.102 ± 0.011) for all melanoma SLN. The mean CXCL12 mRNA copy ratio in H&E/IHC(+) SLN was 0.106 ± 0.020 ($n = 37$). The mean CXCL12 mRNA copy ratio in H&E/IHC(–) SLN was 0.101 ± 0.013 ($n = 93$). There was no significant difference between H&E/IHC(+) SLN and H&E/IHC(–) SLN ($p = 0.20$; Table 2). For H&E/IHC(+) SLN, the mean CXCL12 mRNA copy ratio was significantly lower in macrometastasis(+) SLN (0.065 ± 0.030; $p = 0.02$) than in micrometastasis(+) SLN (0.141 ± 0.028). The mean CXCL12 mRNA copy ratio was significantly higher in micrometastasis(+) SLN (0.141 ± 0.028) than in H&E/IHC(–) SLN (0.101 ± 0.013; $p = 0.04$) (Table 2). This implied that early stages of SLN micrometastasis development can activate chemokine production.

Table 2
CXCL12 and CCL21 Expression in SLN

		CXCL12/GAPDH ratio		*CCL21/GAPDH ratio*	
SLN status	*Number of Specimens*	*Mean±SEM*		*Mean±SEM*	
H&E/IHC met(+)	37	0.106 ± 0.020		2.72 ± 0.29	
			NS		$p = 0.004$
H&E/IHC met(–)	92	0.101 ± 0.013		1.31 ± 0.18	
SLN(micromet)	20	0.141 ± 0.028		3.66 ± 0.37	
			$p = 0.02$		$p = 0.006$
SLN(macromet)	17	0.065 ± 0.030	$p = 0.04$	1.54 ± 0.42	$p < 0.001$
			NS		NS
H&E/IHC met(–)	92	0.101 ± 0.013		1.31 ± 0.18	

NS: not significant

2.2. CCL21 mRNA Expression in SLN

The expression of CCL21 was assessed in SLNs of melanoma patients. The CCL21 mRNA copy ratio in melanoma SLNs ranged from 0 to 10.50 (1.71 ± 0.16). The mean CCL21 mRNA copy ratio in H&E/IHC(+) SLN (2.72 ± 0.29) was significantly higher than in H&E/IHC(–) SLN (1.31 ± 0.18; $p = 0.004$) (Table 2). Among H&E/IHC(+) SLN, the mean CCL21 mRNA copy level ratio was significantly higher in micrometastasis(+) SLN (3.66 ± 0.37; $p = 0.006$) than in macrometastasis(+) SLN (1.54 ± 0.42). In addition, the mean CCL21 mRNA copy ratio was significantly higher in micrometastasis(+) than in H&E/IHC(–) SLN ($p < 0.001$) (Table 2).

These results demonstrate that, in the early development of micrometastasis, CXCL12 and CCL21 increase significantly above levels found in metastasis-free SLNs. As tumor burden within the SLN progressed from micrometastasis to macrometastasis status, the expression of both CXCL12 and CCL21 was suppressed, suggesting that as tumor burden was enhanced within the SLN, there was significant downregulation of CXCL12 and CCL21 expression.

2.3. CXCL12 and CCL21 mRNA Expression in Metastasis-Free SLN

Breslow thickness is a significant prognostic factor in early-stage cutaneous melanoma. To determine whether the downregulation of CXCL12 and CCL21 expression of SLNs is influenced by primary tumor burden, we analyzed CXCL12 and CCL21 expression in metastasis(–) SLNs from tumors with Breslow thickness $\leq$2.0 mm ($n = 55$) versus tumors with Breslow thickness 2.01 to >4 mm ($n = 37$). This analysis allowed us to evaluate the effect of primary melanoma tumor growth to SLN CXCL12 and CCL21 expression. The CXCL12 mRNA copy ratio was significantly lower in SLN of thick primary melanomas (0.077 ± 0.017) compared to that of thin melanomas (0.136 ± 0.021; $p < 0.0001$) (Table 3). Similarly, the CCL21 mRNA copy ratio was significantly lower in metastasis(–) SLN from thick primary melanomas (1.21 ± 0.17) compared to that of thin melanomas (1.51 ± 0.21; $p = 0.032$) (Table 3). These findings suggested that the downregulation of CXCL12 and CCL21 expression in SLN was significantly influenced by increasing primary tumor burden.

2.4. IHC Analysis of Chemokine Expression in SLN

CXCL12 and CCL21 proteins in PE melanoma SLNs were assessed using IHC. Sections (5 μm) were deparaffinized in xylene and then incubated with mouse antihuman CXCL12/SDF-1 monoclonal IgG antibody (10 μg/ml; R&D System, Minneapolis, MN)

Table 3
CXCL12 and CCL21 Expression in H&E/IHC(–) SLN from Thin and Thick Primary Melanomas

		CXCL12/GAPDH ratio		*CCL21/GAPDH ratio*	
	Number of specimens	*Mean ± SE*		*Mean ± SE*	
H&E/IHC(-) SLN					
Thin primary melanoma (≤2.0 mm)	55	0.136 ± 0.021		1.51 ± 0.21	
			$p < 0.0001$		$p = 0.032$
Thick primary melanoma (2.01 to >4 mm)	37	0.077 ± 0.017		1.21 ± 0.17	

or goat antihuman 6Ckine IgG antibody (15 μg/ml: R&D System) at 4°C overnight. For the secondary developing reagents, LSAB+ kit (Dako Corp, Carpinteria, CA) was used. Slides were developed with AEC substrate chromogen (Dako Corp) and were counterstained with hematoxylin *(21)*. The SLNs with high expression of each chemokine mRNA showed strong immunostaining for each chemokine protein, and the specimens with low expression of each chemokine mRNA showed low or no staining of each chemokine protein. IHC analysis demonstrated that chemokine mRNA expression correlated with the presence of the respective chemokine protein.

3. DISCUSSION

The presence of LN metastasis is an important determinant of disease outcome for early-stage melanoma patients. The SLN is the most probable site for the earliest stages of melanoma regional LN metastasis. A H&E/IHC(–) SLN may represent an LN to which tumor cells have not metastasized, or may represent an LN to which tumor cells have metastasized but were destroyed. Similarly, a micrometastasis(+) SLN may not represent a true metastasis. The fates of these metastasized tumor cells are not predestined, and they, too, may undergo spontaneous apoptosis or destruction by immune cells or antibodies if the SLN was not removed. The LN microenvironment can be functionally immune modified by early tumor cell metastasis and play a significant role in determining the cause of metastasis.

CXCR4 and CCR7 are chemokine receptors that are widely expressed on immune cells. CXCR4 expression is important for trafficking and recruitment at sites of tissue injury and inflammation. CCR7 is a major homing receptor of the immune system for trafficking of naïve T cells and DCs. It is known that their respective ligands, CXCL12 and CCL21, play an important role in the recruitment and mounting of immune responses by activation of specific T cells and DCs, respectively. In response to stimulation and/or inflammation, LNS release chemokines to induce migration of activated DCs and naïve T cells bearing chemokine receptors towards the LNs *(7–9)*. Our results demonstrated that melanoma SLNs with micrometastases had the highest level of CXCL12 and CCL21 expression compared to H&E/IHC(–) SLN or SLN macrometastasis(+). These results suggest that micrometastasis promotes activation of chemokine expression within the SLN microenvironment, thereby orchestrating immune cells to the SLN for activating tumor immunity. Given the potential immunogenicity of melanoma cells, activation of immune responses in LNs would occur during the early stages of micrometastasis development.

We hypothesized that increasing melanoma burden in the SLN suppresses chemokine production and creates a more favorable microenvironment for metastasis progression in the SLN. Carriere et al. *(22)* reported, in animal studies, that the recruitment of naïve lymphocytes is impaired in melanoma-draining LN, and this effect is associated with an important defect in lymphocyte adhesion in the HEVs and a progressive decrease in the expression of the LN chemokine CCL21. Our study validates this animal model and demonstrates the significance of this finding in human melanoma metastasis development in regional draining SLNs. The advantage of using patients undergoing SLN dissection is that the SLN is likely to be influenced by the primary tumor is a well defined specimen source. We previously reported in preliminary results that metastasis(+) LNs suppress CCL21 production *(21)*, and our current study demonstrates that metastasis(+) LNs may reduce its production of CXCL12 as well. Increasing tumor burden (macrometastasis) within melanoma SLNs correlates with

downregulation of CXCL12 and CCL21 expression as compared to micrometastasis(+) SLNs. With increasing SLN tumor burden, decreased chemokine expression would prevent the recruitment of immune cells critical for immune surveillance and effective antitumor response, including DCs in activating tumor immunity. Other studies have reported that metastasis to the SLN suppress DCs and T-cell activity *(4,23)*.

The immune modulation of SLN induced by primary tumor-released factors would dampen chemokine expression resulting in a more favorable environment in the LN for metastasis to progress. Increase in metastatic burden will lead to the eventual immune suppression of the SLN and nodal basin and allow tumor metastasis to progress to other LNs *(4)*. We demonstrated that suppression of CXCL12 and CCL21 expression correlated with increasing primary tumor Breslow thickness. To ensure that CXCL12 and CCL21 expression was not influenced by potential tumor burden in the SLN, we analyzed CXCL12 and CCL21 expression; in SLNs (AJCC stages I and II). As primary melanoma progressed, both CXCL12 and CCL21 mRNA expression, in H&E/IHC(–) SLN were downregulated suggesting that the chemokine downregulation of SLN can result from primary tumor growth. This observation is significant in that it may provide an explanation of why increasing Breslow thickness of a primary tumor is associated with a worse prognosis. The downregulation of CXCL12 and CCL21 expression would dampen SLN's ability to direct effective antitumor immune responses. These results suggest that induced chemokine suppression in SLN is not specific to just one chemokine CXCL12 as in other reports. Other chemokines also show upregulation or downregulation of production in SLNs as metastasis develops.

Chemokines are highly critical in orchestrating effective immunity in the LN. Disruption of this response would lead to significant problems in peripheral site disease control. The stromal cells and HEVs in LNs are the primary source of CXCL12 and CCL21 production, respectively. It is apparent that primary melanomas release bioactive factors that induce the downregulation of chemokine production in LNs by inhibiting stromal cells or HEVs' production of chemokines. The mechanism of chemokine regulation in LNs in regional sites during infection, injury, or tumor development remains undetermined. Understanding the regulatory events would be important in developing immunotherapeutics to control disease regional progression. Recently, we and others have demonstrated that when CCL21 is given in conjunction with tumor melanoma vaccine immunization, it significantly augmented tumor immunity *(24,25)*. This supports the significant role of chemokine CCL21 in orchestrating effective tumor immunity. We also reported that administration of CCL21 with a melanoma vaccine activated active-specific immunotherapy more efficiently *(25)*. The studies demonstrated the significant ability of CCL21 in augmenting protective tumor antigen-specific immune responses. These findings suggest that chemokines are be key factors in promoting effective antitumor immunity.

Specific chemokine receptors present on tumor cells respond to their respective chemokines of specific organ sites allowing tumor cells to target these organs preferentially as metastatic sites. The chemokine receptors, CXCR4 and CCR7, are expressed in human cutaneous melanoma *(14,15,21,26,27)*. The respective chemokine ligands, CXCL12 and CCL21, are expressed in the organs to which melanoma cells commonly metastasize to, such as LNs, lung, and liver *(14,21)*. Studies have shown that melanoma cells with CXCR4 or CCR7 respond to organs that produce respective ligands to these chemokines. This chemokine receptor–ligand axis has been suggested to play a role in early stages of metastasis development in the LNs *(26)*. Chemokines are an integral part of the inflammatory response and can function as a double-edged sword: they can enhance tumor immunity and immune surveillance, and at the same time, may promote tumor metastasis *(28)*.

In summary, our studies suggest that increase in SLN metastasis burden and primary tumor progression dampens CXCL12 and CCL21, production which are key chemokines involved in the recruitment of T cells, DCs, and NK cells. Chemokine suppression of LNs may be a significant factor in causing the immune dysfunction of regional tumor-draining nodal basin in preventing metastasis development. Therapeutic agents that can enhance chemokine activity or reverse suppression in LNs may potentially have utility in controling regional melanoma progression.

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21 Circulating and Disseminated Tumor Cells from Solid Tumors—Research and Clinical Aspects

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Contents

ABSTRACT

Increasing evidence indicates that tumor cell dissemination starts already early during tumor development and progression. Sensitive immunocytochemical and molecular assays allow now the detection of single circulating tumor cells (CTC) in the peripheral blood and disseminated tumor cells (DTC) in the bone marrow (BM) as a common and easily accessible homing organ for cells released by epithelial tumors of various origins. Tumor cells are frequently detected in the blood and BM of cancer patients without clinical or even histopathologic signs of metastasis. The detection of DTC and CTC may yield important prognostic information and might help to tailor systemic therapies to the individual needs of a cancer patient. A single DTC or CTC can express properties distinct from that of the primary tumor (e.g., increased rate of HER2/neu expression/amplification), and characterization of DTC/CTC could, therefore, help to identify therapeutic targets and select patients whose tumors are most likely to respond to targeted agents. Moreover, CTC measurements could be used for monitoring the efficacy of systemic therapies. Ongoing clinical trials will reveal whether changes in CTC status will be linked to clinical outcome. Here, we review the data on (i) BM as common homing organ for disseminating tumor cells and

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(ii) clinical studies on disseminating tumor cells that help to establish CTC/DTC measurements in clinical practice, and outline the (iii) biological characteristics of DTC and CTC.

Key Words: epithelial cancers; breast cancer; disseminated tumor cells; circulating tumor cells; bone marrow; blood

1. INTRODUCTION

Despite advances in the treatment of many solid tumors, distant metastases remain a significant cause of cancer-related death. In high-risk disease, early spread of tumor cells is largely undetected even by high-resolution imaging technologies, preventing early potentially effective intervention. Sensitive immunocytochemical and molecular assays now enable specific detection of "occult" metastatic tumor cells even at the single-cell stage, and at a point characterized as minimal residual disease (MRD). These technologies provide the potential to track systemic tumor cell dissemination in the blood and the bone marrow (BM) as one of the first crucial steps in the metastatic cascade. In the clinic, detection and characterization of disseminated tumor cells (DTC) in BM or circulating tumor cells (CTC) in blood could help to guide treatment decisions before the onset of overt metastasis as well as in the setting of advanced disease.

2. BONE MARROW AS COMMON HOMING ORGAN FOR DISSEMINATING TUMOR CELLS

Recent publications have shown that certain properties of the primary tumor enable early hematogenous tumor cell dissemination into the BM *(1,2)*. As an example, tumor cells express the CXC-chemokine receptor on their cell membrane, and it has been reported that metastatic cells may use this chemokine-mediated mechanism to home to specific distant sites (i.e., lung, liver, and BM in breast cancer) *(3)*. Interestingly, BM seems to be a common homing organ for DTC derived from various types of malignant epithelial tumors (e.g., breast, lung, prostate, or colorectal cancer) *(4)*. Many investigators have used cytokeratins as marker antigens; these cytoskeleton proteins are expressed in all epithelial tumors and are usually absent in hematopoietic cells *(4)*. In a large database, DTC were found to be present in BM samples of 20–40% of patients with early-stage breast cancer, even in the absence of lymph node metastases (N_0) or clinical signs of overt distant metastases (M_0) *(5)*. DTC are even detected in the BM of patients who have cancers that do not commonly metastasize to the bone (e.g., colon cancer) *(4)*, suggesting that BM might be a preferred reservoir for these cells.

3. CLINICAL STUDIES ON DISSEMINATING TUMOR CELLS

Clinical studies have shown significant correlations between the presence of DTC in the BM of patients with various tumor types and the risk of eventual metastatic relapse *(4)*, suggesting that founder cells of overt metastases (i.e., "metastatic stem cells") might be a component of DTC. An extensive database is available for early-stage breast cancer *(5)*, linking the persistence of DTC in BM to an increased risk of late metastatic relapse *(4)*. However, it is intriguing that only half of the breast cancer patients with DTC relapsed, whereas the other half remained free of overt metastasis over a 10-year follow-up period following initial diagnosis. This finding is in line with data from animal models and suggests that a significant fraction of DTC never develop into overt

metastases but possibly die or remain in a dormant state. Thus far, little is known about the conditions required for the escape from the dormant or quiescent phase into the dynamic phase of metastasis formation. Meng et al. found CTC in the blood of 13 out of 36 long-term survivors of breast cancer at various times compared with only 1 out of 26 controls. Given the short half-life of these circulating cells, they postulate a possible balance between tumor replication and cell death lasting as long as 22 years following diagnosis of breast cancer *(6)*. This balance might be disturbed by both changes in the DTC (e.g., additional mutations) and the surrounding microenvironment (e.g., decrease in immune surveillance), resulting in the development of metastatic disease.

Peripheral blood is an ideal source for the detection of CTC because of ease of sampling and the ability to sample overtime. Therefore, CTC would be an excellent resource for "real-time" monitoring and perhaps characterization of MRD. Depending on the detection technique used, CTC were detected in 50–100% of patients with metastatic prostate or breast cancer compared with a rate of 10–60% in patients with no clinical signs of overt distant disease *(7)*. Most of these CTC are indeed viable, as shown by a new technique designated "EPISPOT assay" for EPithelial ImmunoSPOT *(8)*. This technique is a protein-secreting profiling based on the secretion or active release of specific marker proteins using an adaptation of the enzyme-linked immunospot technology. This allows the specific detection of viable cells after a $CD45^+$ cell depletion and was introduced for the purpose of DTC/CTC analyses from BM aspirates and blood samples *(9)*. In a prospective, multiinstitutional clinical trial, detection of CTC with the FDA-approved CellSearch ™ system provided significant prognostic information before and also early (4 weeks) after initiation of chemotherapy in patients with measurable metastatic breast cancer *(10)*. Early reduction in CTC count at first follow-up after treatment change to an absolute number below the specified threshold value was associated with improved disease-free and overall survival compared with patients whose CTC counts failed to decrease. In fact, the posttreatment count appeared to be a more significant predictor of outcome than the baseline pretreatment values. In contrast, the prognostic relevance of CTC in the blood of patients with early-stage disease is still under intense investigation *(11)*.

4. BIOLOGICAL CHARACTERISTICS OF DISSEMINATED AND CIRCULATING TUMOR CELLS

Various studies have revealed a striking heterogeneity of DTC and CTC with regard to the expression of growth factor receptors, proteases, adhesion molecules, and major histocompatibility complex antigens as well as cytogenetic aberrations *(4)*. Among the protein characteristics, expression of the tyrosine kinase receptor HER2/neu on DTC appears to be linked to metastatic relapse in a heterogeneously treated population *(4)*. Functional studies on DTC and CTC are difficult because of their low frequency (i.e., 1×10^{-5}–10^{-6}) in BM and blood, respectively. However, data suggest that more than 60% of patients with various tumor types (colon, prostate, breast, and renal cell cancer) harbor BM DTC that grow transiently under optimized in vitro conditions, and a strong growth potential of these cells has been associated with poor clinical outcome *(12)*. Interestingly, epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2)—two known stem cell growth factors—were relevant for the in vitro growth of DTC obtained from BM of cancer patients. Since the growth capacity of these cells was correlated with clinical outcome *(12)*, it is possible to hypothesize that EGF and FGF-2 are important for the formation of solid metastases in cancer patients. In this context, it is noteworthy that a subset of circulating prostate cancer cells in the peripheral blood can secrete FGF-2

(8). Future clinical follow-up studies will show whether patients with these particular cells have an increased risk of metastatic relapse specifically in the bone or BM, the most frequent site of metastases in prostate cancer.

A recent small study found that a high percentage of DTC in the BM of patients with breast cancer express a $CD44^{+}CD24^{-/low}$ phenotype, previously shown to represent a minor population in primary breast cancer with high self-renewal and tumorigenic potential *(13)*. Moreover, studies using the EPISPOT assay found that a viable subpopulation of DTC in BM had the $CK19^{+}MUC1^{-}$ phenotype, previously also suggested as a breast stem cell marker *(8,14)*. We, therefore, propose that a fraction of DTC in early-stage cancer patients could represent putative metastatic stem cells. This provocative hypothesis is in line with the fact that most DTC in the BM (and CTC in the peripheral blood) are nonproliferating (i.e., Ki-67 negative) *(15,16)* and resistant to chemotherapy *(15,17)*, as postulated for cancer stem cells. Nevertheless, it is still debated whether cancer stem cells are circulating via the blood to distant organs and whether BM is a preferred homing organ for DTC with stem cell properties.

5. CONCLUSIONS

Final consensus is now needed regarding quality control issues and criteria for acceptable technical assay performance. Future research on DTC/CTC should lead to increased understanding of the metastatic cascade in cancer patients. Evolving research topics in this field include the regulation of tumor dormancy and identification of metastatic stem cells, which could eventually result in the development of new therapeutic approaches to prevent metastatic relapse, as well as early identification of patients at highest risk for relapse and death. A single DTC or CTC can express properties distinct from that of the primary tumor (e.g., increased rate of HER2/neu expression/amplification) *(4)*. Characterization of DTC/CTC could, therefore, help to identify therapeutic targets and select patients whose tumors are most likely to respond to targeted agents. In addition, CTC measurements could be used for monitoring the efficacy of systemic therapies. The development of an improved platform of DTC/CTC detection incorporating the new discoveries on the biology of early tumor cell dissemination is the main focus of the current European project group DISMAL (Disseminated Malignancy; www.dismal-project.eu). A randomized clinical trial in the metastatic setting is testing the impact of changing systemic therapy based on CTC response compared with using standard imaging and clinical parameters.

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22 Head and Neck Cancer: An Example for the Role of Chemokine Receptors in Tumor Progression and Metastasis

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ABSTRACT

Despite ongoing advances in cancer therapy, a large number of patients are still afflicted with metastatic disease, the most lethal aspect of human cancer. Thus, understanding the factors that promote tumor metastasis is a critical area in cancer research. Recent findings demonstrate that tumor cells adopt chemokine receptor-mediated pathways from leukocytes to perform organ-specific metastasis. Here, we summarize recent advances in head and neck cancer (HNC) and describe the role of chemokine receptors in tumor progression and metastasis.

Key Words: chemokine; chemokine receptor; head and neck cancer; invasion and metastasis; CXCR4; CCR7

1. INTRODUCTION

Despite ongoing advances in cancer therapy, a large number of patients are still afflicted with metastatic disease, the most lethal aspect of human cancer. Thus, understanding the factors that promote tumor metastasis is a critical area in cancer research. Mechanisms of metastasis have

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received scientific investigation for over 100 years since publication of the "seed and soil" hypothesis by Stephen Paget in 1889, which described metastasis for the first time as a nonrandom process *(1,2)*. Indeed, it has been observed clinically that certain tumor types (the seed) show tissue tropism for particular organs (the soil) where they establish sites of malignant growth *(2)*.

Head and neck carcinomas (HNCs) comprise tumors with different histological phenotypes and distinct clinical characteristics. Squamous cell carcinomas (SCCs) of the upper aerodigestive tract mucosa are the most common histological subtype *(3)*. Adenoid cystic carcinoma (ACC) is a rare malignant epithelial tumor arising from salivary glands *(4)*. Both tumor entities are characterized by distinct metastatic patterns. While SCC frequently metastasizes to regional lymph nodes *(5)*, ACC is more aggressive, disseminating to distant sites, particularly the lung and liver *(6,7)*. In both these tumor types, current treatment regimens result in fairly good locoregional control, but little progress has been made in the treatment of disseminated disease (Fordice, 1999 p. 161; Mao, 2004 p. 180; Vokes, 1993 p. 472). Hence, metastatic spread represents an important survival-limiting factor.

Due to their distinct metastatic patterns, ACC and SCC provide an interesting model to investigate the molecular mechanisms of organ-specific metastasis.

Recent studies have demonstrated that tumor cells express distinct patterns of chemokine receptors *(8–11)*. These receptors regulate cellular functions associated with tumor progression and metastasis in vitro and in vivo *(12)*.

2. CHEMOKINE RECEPTORS

Chemokine receptors represent pertussis toxin-sensitive, 7-transmembrane-spanning G-protein-coupled receptors (GPCRs) *(13,14)*. To date, 10 CC-chemokine, 7 CXC-chemokine, 1 CX3C, and 1 XCR-receptors have been characterized *(13,14)*. Chemokine receptor signaling involves different pathways sustaining cell survival, inducing gene expression, and most importantly enabling directional cell migration *(13,14)*. Although the family of their ligands is most likely complete, several "orphan" GPCRs may represent putative partners.

Chemokine receptors bind small, cytokine-like peptides/proteins (8–14 kDa) that selectively attract and activate different cell types *(13,14)*. Chemokines are well known to play a pivotal role in leukocyte recruitment and organize innate as well as adaptive immune responses *(13,14)*. The chemokine superfamily (45 members in the human) is thought to be among the first functional protein families completely characterized at the molecular level. This offers a unique opportunity to systematically characterize their involvement in physiological and pathophysiological conditions. Chemokines can be divided into four subclasses based on the arrangement of the cysteine residues at the amino terminus: CXC chemokines (termed as CXC ligands, CXCL), CC chemokines (CCL), C chemokines (XCL), and CX3C chemokines (CX3CL) *(13,14)*.

Interestingly, there is a certain degree of promiscuity in the chemokine superfamily with many ligands binding different receptors or vice versa. So-called "cluster" chemokines representing chemotactic proteins, which share a distinct chromosomal location, are likely to bind the same receptors *(13)*. However, "noncluster" or "microcluster" chemokines are ligands, which demonstrate a unique chromosomal location and tend to present a restricted or even specific chemokine receptor interaction *(13)*.

During recent years, studies using gene-deficient mice or neutralizing antibodies demonstrated that the set of noncluster or microcluster chemokines encodes for nonredundant biological functions *(15–17)*. They critically regulate biological processes such as organogenesis and stem cell homing to the bone marrow (CXCL12/CXCR4) *(18–23)*, naïve T-cell homing to the lymph

node (CCL21/CCR7) *(15,24,25)*, B-cell homing to the B-cell follicles (CXCL13/CXCR5) *(26–28)* as well as organ-specific recruitment of effector memory T cells to the gut (CCL25/CCR9) *(29–31)* or to the skin (CCL27/CCR10) *(32–34)*.

The formation of metastases is thought to be the result of several sequential steps that share many similarities with leukocyte trafficking, a process critically regulated by chemokines and their receptors *(35)*. During the multistep process of leukocyte trafficking, chemokine ligand–receptor interactions mediate the firm adhesion of leukocytes to the endothelium and initiate transendothelial migration from the blood vessel to the perivascular pockets. From perivascular spaces matrix-bound sustained chemokine gradients direct infiltrating leukocyte subsets to sublocations or intramucosal locations. Likewise circulating tumor cells need to interact with the vascular endothelium and they depend on extravasation and invasion to establish metastatic tumor foci in distinct peripheral organs.

Hence, we hypothesized that tumor cells adopt chemokine receptor-driven pathways from leukocytes to establish organ-specific metastases.

3. CHEMOKINE RECEPTOR EXPRESSION IN HEAD AND NECK CANCER

The presence of local and distant metastases is a key criterion of the malignant phenotype and represents an important prognostic as well as survival-limiting factor for most cancer types. In general, cancer cells metastasize to distinct organs in a nonrandom manner. To investigate cellular characteristics that may be important in the process of organ-specific metastasis, we selected two different types of HNC: SCC, characterized by frequent presence of lymph node metastases *(5)*, and ACC, a rare cancer type characterized by hematogenous dissemination *(6,7)*. These two tumor entities serve as a model to study the organ specificity of the metastatic process.

In two independent studies, we addressed the chemokine receptor repertoire of HNC cell lines as well as primary tumors and metastases *(36–38)*. The first study focused on SCC of the head and neck region *(37,38)*.

Here a selective upregulation of CCR7 in metastatic SCCHN cell lines and tumor tissues was demonstrated *(37)*. Corroboration of these data using immunohistochemical staining as well as quantitative reverse-transcription (qRT)-polymerase chain reaction (PCR) on paired tumor biopsies (primary and metastatic) indicated significantly higher expression of CCR7 on metastatic tumors as compared with primary tumor tissue *(37)*. The study also compared other chemokine receptor expression profiles (CCR1 to CCR10 and CXCR1 to CXCR5) between our primary tumor and metastatic SCCHN cell lines *(37)*. These findings do not exclude the concomitant expression of other chemokine receptors in addition to CCR7, although none of these has demonstrated a consistent pattern of expression that distinguishes the metastatic from primary tumors *(37)*. Notably, despite reports studying the role of CXCR4 (another nodal homing chemokine receptor) in SCCHN *(39,40)*, we have not detected any appreciable or consistent expression of this receptor on our SCCHN cell lines or tumor tissues *(37)*. Also, recent evidence indicates that CCR7 appears to be a stronger mediator of lymph node homing for dendritic cells (DCs) than CXCR4 *(41)*. This may also hold true in the case of SCCHN tumor cell migration during nodal invasion and metastasis. CCR7, a seven-transmembrane-domain GPCR, is important for immune cell trafficking and homing to the lymph nodes, and is expressed on mature DCs, B cells, naïve, and some memory T cells. Its ligands, CCL19 and CCL21, are expressed minimally by stromal cells in lymphoid organs and regulate the recruitment and homing of CCR7 positive ($CCR7^+$) cells to lymphatic tissue. While chemokine receptors were initially described and identified on immune cells, it is increasingly apparent that functional

chemokine receptors are expressed by some endothelial cells *(42)* and transformed epithelial cells *(8)*. Malignant cells that express functional chemokine receptors can respond to environmental cues intended for immune cells, migrate along chemokine gradients to distant organs, and establish sites of secondary tumor growth.

The second study performed a direct comparison between SCC and ACC of the head and neck region and demonstrated a distinct chemokine receptor expression profile for each HNC entity *(36)*. SCC cells expressed a broad variety of chemokine receptors. The majority of SCC cell lines, similar to mucosal keratinocytes, expressed significant levels of CXCR1 and CXCR2 transcripts *(36)*. These two chemokine receptors have been associated with CXCL8-mediated proliferation of melanoma and colon cancer cells *(43,44)*. Hence, it is conceivable that CXCL8, also produced by SCC cells themselves *(45)*, may promote the proliferation and invasion of SCC cells expressing CXCR1 and CXCR2. In SCC cell lines, we consistently observed upregulation of CCR7 and CXCR5 mRNA, while mRNA for these receptors was absent or expressed at low levels in normal mucosal keratinocytes and ACC cells *(36)*. Interestingly, the corresponding ligands of these receptors (CCL19, CCL21, and CXCL13, respectively) are homeostatically expressed in lymph nodes *(46–48)*. CCR7 expression is critical for the migration of naïve lymphocytes and mature DCs to draining lymph nodes *(35)*. Furthermore, CCR7 expression has been associated with lymph node metastasis in several other cancer types, such as melanoma, lung cancer, gastric cancer, and esophageal cancer *(49–52)*.

In our panel of SCC cell lines, CCR7 protein was only detectable intracellularly, whereas in tissues, surface expression was evident in primary tumors examined by immunohistochemistry *(36)*, suggesting that the transport of CCR7 protein to the cell surface may require microenvironmental factors present only in vivo. Indeed, our previous observations show that IL-1β, tumor necrosis factor (TNF)-α, or epidermal growth factor (EGF) induce relevant chemokine receptors on the surface of melanoma cells (unpublished data). However, no surface CCR7 expression was detected in SCC cells either after serum starvation or after treatment with inflammatory cytokines or growth factors *(36)*, suggesting that either a combination of factors or yet unidentified compounds are required for the recruitment of CCR7 to the surface of SCC cells.

The majority of primary tumors and lymph node metastases of SCC expressed CCR7 *(36)*. Thus, CCR7 might play a role in directing SCC cells to the draining lymph nodes and contribute to the frequent presence of lymph node metastases in SCC patients, as also suggested by Wang et al. *(37)*. It has been proposed that SCC cells of the head and neck region mimic the differentiation and migration pattern of DCs displaying a $CCR6^{+}CCR7^{-}$ phenotype within the skin and a $CCR6^{-}CCR7^{+}$ phenotype on their way to local draining lymph nodes *(37)*. However, in our study, no differences in CCR7 expression could be observed neither in matched primary SCC cell lines at the mRNA level nor in tissue sections of primary SCC tumors and matching lymph node metastases at the protein level *(36)*. Our results are in accordance with recent results of Ding and coworkers *(50)* demonstrating CCR7 expression in both primary tumors and lymph node metastases of esophageal SCC. These observations are in line with recent studies demonstrating that primary tumors are similar to the corresponding metastatic tumors in their gene expression signature *(53,54)*. Thus, regulation of chemokine receptors may be a relatively early event during tumorigenesis.

CXCR5 participates in the trafficking of B and T cells to secondary lymphoid organs *(46,47)*. Recently, it has been suggested that in leukemic B-cell lymphomas CXCR5 may be associated with the spread of malignant B cells to lymphoid tissues *(55,56)*. Here, we show the abundant expression of CXCR5 transcripts and the presence of surface CXCR5 on SCC cells *(36)*. Our observation is consistent with the concept that metastasis and leukocyte trafficking share underlying mechanisms and suggests that CXCL13/CXCR5 interactions may provide a novel complementary pathway mediating lymphogenic spread.

The only chemokine receptor found to be highly expressed in ACC but not in SCC was CXCR4 *(36)*. In ACC cells, CXCR4 signaling resulted in the induction of directional tumor cell migration supporting its role in tumor invasion and metastasis *(36)*. Recent studies uncovered the fundamental role of CXCL12/CXCR4 interactions in physiological processes, such as organogenesis, hematopoiesis, and homing of progenitor cells to liver and bone marrow *(57)*. In addition to its complex physiological functions, CXCR4 has been shown to be pivotal during the metastatic spread of tumor cells to distant organs *(8,10,58–60)*. The abundant expression of CXCR4 in the predominantly hematogenously metastasizing ACC suggests that CXCR4 may play a role in directing ACC cells to their metastatic sites. ACC most frequently metastasizes to the lung and liver *(6)*, and CXCL12, the only known CXCR4 ligand, is abundantly expressed in these organs *(8)*. These data suggest that CXCL12/CXCR4 interactions play a crucial role in hematogenous spread of tumor cells. In contrast to the frequent hematogenous dissemination, lymphogenous metastases in ACC are extremely rare *(61)*, despite the fact that lymph nodes are also an abundant source of CXCL12. In lymph nodes, CXCL12 is expressed by stromal cells in close vicinity to high endothelial venules representing the blood–lymph node interphase *(62)* (unpublished observations). Recent data suggest that CXCL12 expressed in lymph nodes contributes to the homing of memory T cells to lymph nodes via the bloodstream but not the lymphatics *(63)*. Hence, the microanatomical distribution of CXCL12 in lymph nodes may partially explain why lymphogenous metastasis is not observed in ACC with abundant CXCR4 expression. Although several studies have suggested a role for CXCR4 in lymph node metastasis, accumulating evidence suggests that CXCR4 is not a major participant in lymphatic tumor spread, including large-scale gene expression data in metastatic head and neck SCC *(64)*. Moreover, extravasation requires a complex interplay of chemokine receptors, adhesion molecules and other factors, and ACC cells may be deficient in some factors that are essential for extravasation or other steps in the process of lymphogenous metastasis.

In addition to ACC, a subset of SCC tumors also demonstrated CXCR4 expression in vivo *(36)*, which is in accordance with recent studies *(65–67)*. Notably, two of the CXCR4-expressing SCC tumors showed enhanced CXCR4 expression at the leading edge of the tumor *(36)*, suggesting a possible role for this receptor in primary tumor invasion, as it also has been suggested in prostate and ovarian cancer *(68,69)*.

These findings are in line with observations by others showing that a murine B16 melanoma model, transfected with CCR7, enhanced metastases to the regional lymph nodes *(49)*. Similarly, other studies with transfection of CXCR4 led to enhanced lung metastasis *(70)*, while CCR10 was associated with dermal metastasis *(71)*.

4. CHEMOKINE RECEPTORS MEDIATE TUMOR CELL MIGRATION, INVASION, AND SURVIVAL

When chemotactic ability was tested in an in vitro transwell migration assay, metastatic SCCHN tumor cells migrated selectively in response to CCL19 and CCL21 at levels comparable to mature $CCR7^+$ DCs *(38)*. Autologous primary tumor cells demonstrated no significant chemotactic response to the two CCR7 ligands *(38)*. Blockade of CCR7 using a monoclonal antibody (mAb) or truncated CCL21 abolished the observed chemotactic migration of metastatic SCCHN cells, indicating specificity of the response to CCL19 and CCL21 *(38)*.

These findings have two major implications. First, the expression of functional CCR7 exclusively by metastatic SCCHN tumors suggests that these tumor variants are capable of responding to chemokine gradients within the tumor microenvironment and migrate to regional nodal tissue where they can establish secondary growth sites. This is consistent with the observed pattern of

lymph node metastasis in SCCHN patients *(5)*. Second, CCR7 has emerged as an important marker of the metastatic capability of SCCHN tumors. High CCR7 expression levels are likely to correlate with a higher metastatic potential and therefore a more aggressive tumor variant. To address this possibility, we compared the level of CCR7 expression between highly metastatic SCCHN cell lines, produced by serial passages of human SCCHN nodal metastases in a nude mouse model *(72–74)* with their poorly metastatic parental cell line. In 3/3 cell isolates studied, the highly metastatic tumors had approximately tenfold higher levels of CCR7 expression by qRT-PCR than the parental metastatic tumor cells obtained from human lymph node, and 100-fold higher levels than the cell line derived from the same patient's primary tumor. These studies were validated by the observed cross-reactivity of murine CCR7 ligands for human CCR7 *(75,76)*. However, because metastatic variants in nude mice could be a result of a number of oncogenic gene products, we have recently used another murine tumor system to investigate the importance of CCR7 in tumor formation.

The discovery of paucity of lymph node T cells (*plt*) mice has greatly advanced our ability to study CCR7 and its ligands in vivo. In wild-type Balb/c mice the CCR7 ligands CCL19 and CCL21 are constitutively expressed and only differ in their spatial distribution. The *plt* mutation results in a loss of expression of CCL19 and CCL21-ser in the secondary lymphoid organs, and therefore an inability to recruit $CCR7^+$ T cells, B cells, and activated DCs into the LN, spleen, and PP *(16,24,77)*. *Plt* mice, therefore, provide an excellent system to study the requirement for CCR7-mediated signaling in SCCHN tumor formation and metastasis. When wild-type Balb/c mice were implanted with a syngeneic murine oral SCC cell line (B7E3), we observed a significantly higher rate of tumor growth and a higher ultimate tumor burden, as compared with their *plt* littermates (unpublished observations). Overexpression of CCR7 partially overcame this difference in *plt* mice, leading to recovery of some tumor formation. These results show that the absence of a chemokine gradient and prosurvival signals to which $CCR7^+$ tumor cells respond can result in an overall decrease in tumor formation. Presumably, the residual levels of CCL21-leu expressed in *plt* mice are still sufficient to support some tumor formation. Taken together, these results suggest that CCR7-mediated signaling may be important not only for the chemotactic migration of invasive $CCR7^+$ tumor cells but also for the survival of growing tumors. In general, elevated CCR7 ligand expression has been associated with the presence of $CCR7^+$ tumors *(38,78)*.

CXCL12, the corresponding ligand of CXCR4, is highly expressed in the lung and liver, organs that represent major destinations of ACC metastasis *(8,61)*. Consequently, we asked whether CXCR4 expressed in ACC cells is functionally active upon ligand binding. When the highly CXCR4-expressing ACC cells were incubated with various concentrations of recombinant human CXCL12, binding of the ligand and time-dependent internalization of the chemokine receptor were seen by flow cytometry *(36)*. Furthermore, a transwell migration assay was performed to examine the effect of CXCL12 on migration of ACC and SCC cells. ACC cells were able to migrate toward CXCL12 in a dose-dependent manner. In contrast to ACC, CXCR4-negative SCC cells did not show a chemotactic response to CXCL12 gradients. Similarly, SCC cells expressing low levels of CXCR4 also did not show significant migration toward CXCL12 gradients *(36)*.

Recent evidence has reported CCR7 signaling to be crucial for providing prosurvival signals to activated DCs and effector $CD8^+$ T cells through the phosphoinositide-3 kinase (PI3K)/Akt pathway *(79,80)*. We examined the possibility of CCR7-induced protection from apoptosis in metastatic SCCHN tumors. Blockade of the EGF receptor (EGFR), another major survival pathway in SCCHN, using either the extracellular blocking mAb C225 or the tyrosine kinase inhibitor AG1478, had no observable effect on CCR7-induced pAkt activation *(81)*. These findings rule out the possibility that increased EGFR ligand secretion or intracellular "cross-talk" between EGFR and CCR7 pathways may be responsible for mediating these prosurvival signals. Thus, the CCR7-induced prosurvival pathway appears to be EGFR independent.

The induction of apoptosis in tumor cells is a major goal of cancer chemotherapy. Cisplatin, a chemotherapeutic agent used in the therapy of HNC, exerts its cytotoxic effect by forming DNA adducts, which in turn activate a complex network of pathways that finally culminate in apoptosis *(82)*. However, following cisplatin exposure, prosurvival pathways are also activated *(82,83)*. The fate of a cell is determined by the balance between proapoptotic and prosurvival pathways. In the present study, we demonstrate that sublethal doses of cisplatin induced CXCR4 on the surface of malignant cells *(36)*. In the presence of the transcriptional inhibitor α-amanitin, the upregulation of CXCR4 by cisplatin was markedly repressed, suggesting that gene transcription is required for this induction. Moreover, we show that in ACC cells CXCL12 stimulation resulted in the activation of Akt and ERK1/2 *(36)*, MAP kinases which are involved in signal transduction pathways generally associated with cell survival and proliferation *(84,85)*. Our findings are supported by recent observations showing that CXCL12 can induce survival and proliferation signals in several cell types, such as $CD4^+$ T cells *(86)*, embryonic neural cells *(87)* as well as cancer cells including breast cancer, pancreatic cancer, and glioblastoma *(10,88–90)*. In addition to the activation of survival pathways, we also provide evidence that CXCL12 reduces the rate of apoptosis induced by cisplatin in ACC cells. We propose that suppression of apoptosis via CXCR4 signaling may lead to increased tumor cell viability and might contribute to cisplatin resistance and the failure of antineoplastic treatment of metastatic ACC.

5. CCR7 DOWNSTREAM ACTIVATION OF NUCLEAR FACTOR-κB COMPLETES THE AUTOCRINE LOOP

We noted that untreated cells are able to sustain CCR7-mediated survival even in the absence of exogenous ligand *(91)*. We assayed cell culture supernatants by ELISA for the secretion of chemokine ligands by our SCCHN cell lines. A matching pattern of ligand secretion was found in which metastatic cell lines expressing CCR7 secrete CCL19, which was confirmed by qRT-PCR both in cell lines and in tumor tissues (unpublished observations). Furthermore, ligand secretion in metastatic SCCHN cell lines is functionally significant, as observed by higher basal levels of phosphorylated Akt in untreated cells compared with cells pretreated with a CCR7-blocking mAb. This ligand secretion pattern suggests that the existence of an autocrine (and paracrine) signaling loop in which chemokine receptor-expressing tumors are capable of secreting and responding to their cognate ligand. This autocrine and paracrine production of chemokines by tumors are similar to that described in DCs *(92)*, and can contribute to the malignant transformation of tumor cells and subsequently their propensity for metastasis by providing self-sustaining growth and survival signals. Autocrine signaling in tumors may also play a role in the selection of clinically aggressive tumor variants. The PI3K/Akt signaling pathway has been reported to lead to the activation of nuclear factor (NF)-κB, a critical regulator of inducible gene expression *(93,94)*.

To determine whether CCR7 stimulation by its ligand results in the activation of NF-κB in a metastatic SCCHN cell line, we treated cells with CCL19 in the presence or absence of CCR7-blocking antibody and quantified NF-κB activation in nuclear extracts using an electrophoretic mobility shift assay (EMSA). CCL19 stimulation was found to activate NF-κB at a level weaker than that induced by TNF-α. This was specific to the CCR7 receptor as indicated by the ability of CCR7-specific mAb to block NF-κB activation. The use of an irrelevant ligand (CCL20) did not activate NF-κB. Furthermore, binding could be competed away by the addition of excess unlabeled but not an irrelevant probe, showing specificity of NF-κB binding. We have also identified NF-κB sites in the CCR7 promoter with transcriptional activity after inflammatory ligand treatment.

Our proposed model for autocrine, NF-κB-mediated CCR7 activation entails CCR7 activation by cognate ligand resulting in the phosphorylation and activation of adjacent G-proteins. Release of the heterotrimeric subunits from the $G\alpha_i$ subunit signals for activation of PI3K via rho GTPases. PI3K then phosphorylates membrane-bound phosphatidylinositol-4,5-biphosphate (PIP_2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP_3). This creates at the plasma membrane, high-affinity docking sites for Akt through its pleckstrin homology (PH) domain. Anchoring of Akt to the plasma membrane allows its phosphorylation and activation by phosphoinositide-dependent kinases at the critical residues Thr^{308} on the kinase domain and Ser^{473} on the hydrophobic motif. Activated Akt is then available to activate the IκB kinases (IKK), which in turn phosphorylate IκB and target it for proteasomal degradation. This allows for the release and subsequent activation of NF-κB which translocates to the nucleus and induces gene expression at the CCR7 locus. The resulting CCR7 gene expression may support enhanced surface CCR7 receptor levels and/or ligand-induced receptor activation. Consistent with this hypothesis, we have confirmed the presence of κB sites in the CCR7 promoter (*Hinz et al., 2002[95],* p. 464) and unpublished observations). Further studies comparing the activation and regulation of CCR7 in tumor cells and DCs will provide useful information on the potential role of inflammatory chemokine signals in these cell types.

6. CONCLUSION

Taken together, findings of recent studies demonstrate that tumor cells express a distinct set of chemokine receptors that mediate tumor cell migration, invasion, and survival. These receptors play a role in tumor progression and metastasis and may represent interesting prognostic markers. Currently, intense efforts are underway to identify small-molecule antagonists for chemokine receptors that could be useful for treating disseminated cancer. Indeed the first generation of these compounds is currently approved for the treatment of nonmalignant diseases. It appears that the efficacy of conventional chemotherapeutic treatment might be enhanced by the combination with chemokine receptor neutralization to repress prosurvival pathways and enhance tumor cell apoptosis. These and other strategies based on emerging mechanistic data represent some of the promising new directions in the therapy of metastatic disease.

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23 Tumor and Lymph Node Lymphangiogenesis

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Contents

ABSTRACT

Metastatic spread to lymph nodes represents the first step of dissemination for the majority of human cancers and serves as an important prognostic parameter. It has been recently found—in experimental tumor models—that tumors can actively induce lymphangiogenesis, the growth of tumor-associated lymphatic vessels, and that tumor lymphangiogenesis promotes metastasis to sentinel lymph nodes. The major tumor lymphangiogenesis factors involved are vascular endothelial growth factor (VEGF)-C, VEGF-D, and VEGF-A. Importantly, clinical studies have confirmed the correlation of tumor lymphangiogenesis and metastasis in many different types of human cancers. Our recent findings indicate that tumors also induce lymphangiogenesis in sentinel lymph nodes, often before the actual onset of metastasis, promoting tumor dissemination to distant lymph nodes and beyond. Based on these findings, lymphangiogenesis has become a new prognostic indicator and also a target for the prevention and treatment of lymphatic metastases.

Key Words: Cancer; lymphangiogenesis; lymphatic metastasis; VEGF-A; VEGF-C; VEGFR-3; angiogenesis

1. INTRODUCTION

The lymphatic vascular system drains interstitial fluid, a protein-rich exudate from the blood capillaries, from the peripheral tissues to the venous circulation. Lymphatic fluid is taken up by lymphatic capillaries, drained to the collecting lymphatic vessels, and returned to the blood circulation via the thoracic duct. Lymphatic capillaries differ from blood vessels by the lack of a basement membrane and of coverage by smooth muscle cells or pericytes *(1)*. Lymphatic

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endothelial cells (LECs) are anchored to the extracellular matrix by anchoring filaments. Thus, increased tissue fluid pressure leads to expansion of lymphatic vessels to enhance their absorptive function *(2)*.

Recently, a number of growth factors have been identified that promote lymphatic vessel growth. The best-characterized lymphangiogenic factors are vascular endothelial growth factor (VEGF)-C and VEGF-D, predominantly acting via VEGF receptor-3 (VEGFR-3) expressed on LECs in normal tissues *(1)*. However, after proteolytic processing, both factors also efficiently bind to VEGFR-2. More recently, VEGF-A has also been found to promote lymphatic hyperplasia, in part by direct interaction with VEGFR-2 *(3–6)*. Moreover, insulin-like growth factors *(7)*, hepatocyte growth factor *(8)*, fibroblast growth factor-2 *(9–11)*, and platelet-derived growth factors *(12)* enhance lymphangiogenesis. Adrenomedullin, which exerts its effects through calcitonin receptor-like receptor and receptor activity-modifying protein-2, also promotes lymphatic vessel growth *(13)*. In addition, angiopoietin-1, acting via the endothelial receptor Tie2 *(14)*, promotes lymphangiogenesis in vivo *(15,16)*.

2. TUMOR LYMPHANGIOGENESIS PROMOTES LYMPH NODE METASTASIS

Lymphatic metastasis has generally been considered as a passive process, and the prevailing hypothesis has been that invasive tumor cells randomly get in contact with lymphatic vessels and are then drained to sentinel lymph nodes. The poor knowledge about the mechanisms of lymphatic metastasis has been due to the lack of reliable markers to distinguish lymphatic from blood vessels, the lack of identified specific lymphatic growth factors, as well as the absence of suitable experimental metastasis models. However, a number of lymphatic vessel-specific markers have been recently identified, and experimental studies in genetic mouse models have revealed that several growth factors are able to promote lymphatic vessel growth and function *(17)*. These discoveries have enabled new experimental approaches toward the elucidation of the mechanisms of lymphatic cancer metastasis—leading to the surprising finding that tumor-induced lymphatic vessels play an active role in promoting cancer metastasis to lymph nodes. Thus, experimental studies in mice revealed that overexpression of VEGF-C or VEGF-D by tumor cells promote tumor lymphangiogenesis and lymph node metastasis *(18–21)*. Moreover, we found that transgenic overexpression of VEGF-A or VEGF-C in epidermal keratinocytes of the skin promotes tumor lymphangiogenesis and lymph node metastasis in a chemically induced multistep skin cancer model *(22,23)*. Whereas, VEGF-A promoted both tumor angiogenesis and lymphangiogenesis, as well as tumor growth, VEGF-C predominantly enhanced tumor lymphangiogenesis without any promoting effect on tumor growth rates. Nevertheless, both factors efficiently enhanced tumor metastasis to lymph nodes, demonstrating the active role of tumor-associated lymphatic vessel growth in cancer dissemination to the draining lymph nodes *(22,23)*.

Based upon these findings, attempts have been made to block lymphatic tumor metastasis by interference with the VEGFR-3 pathway. Indeed, systemic treatment of mice with a VEGFR-3-blocking antibody inhibited lymph node metastasis in a breast carcinoma model *(24)* and also in an orthotopic model of stomach cancer *(25)*. Moreover, delivery of a soluble VEGFR-3 fusion protein to mice reduced the formation of tumor lymphangiogenesis and inhibited lymph node metastasis of experimental melanomas, prostate cancers, breast cancers, and lung cancers in mice *(18,26,27)*. Therefore, tumor lymphangiogenesis has emerged as a novel mechanism that promotes cancer cell dissemination to lymph nodes. Recent evidence indicates that the immunohistological detection of lymphovascular invasion by tumor cells, using stains for lymphatic endothelial markers such as podoplanin or lymphatic vessel hyaluronan receptor-1 (LYVE-1), might also represent a sensitive prognostic indicator for lymphatic cancer spread.

3. A NEW CONCEPT OF TUMOR METASTASIS: LYMPH NODE LYMPHANGIOGENESIS

In addition to lymphangiogenesis induced at the primary tumor site, we have recently found that disseminated, VEGF-A-expressing tumor cells potently induce lymphangiogenesis within metastatic lymph nodes *(23)*. These newly formed lymphatics actively proliferate and express several lymphatic specific markers including the transcription factor Prox1 *(23)*. These findings were made in a multistep skin carcinogenesis model in transgenic mice with epidermis-specific overexpression of VEGF-A. A major new finding of this study was that lymphatic vessel expansion in draining sentinel lymph nodes was already induced before the skin cancers had metastasized *(23)*. More recently, we also found premetastatic lymph node lymphangiogenesis in a skin carcinogenesis study applied to transgenic mice with epidermis-specific overexpression of VEGF-C *(22)*. Together, these results provide a new twist to the century-old seed-and-soil hypothesis *(28)* and indicate that cancers can prepare a premetastatic niche within the draining lymph nodes. The creation of the premetastatic niche involves lymphatic vessel expansion within lymph nodes, most likely mediated by drainage of VEGF-A or VEGF-C by lymphatic vessels from the primary tumor site (Fig. 1). The promotion of lymphangiogenesis is further enhanced once metastatic tumor cells have settled within the draining lymph node. In VEGF-C transgenic mice, the extent of lymph node lymphangiogenesis was correlated with enhanced tumor metastasis to the distant lymph nodes and to the lungs *(22)*.

Together, these results indicate that lymph node lymphangiogenesis represents a newly identified mechanism by which tumor cells can actively promote their metastatic behavior. Importantly, we have recently found that lymph node lymphangiogenesis also occurs in metastatic sentinel lymph nodes of patients with malignant melanoma of the skin *(29)*. Moreover, expansion of lymphatic vessels within sentinel lymph nodes has also been observed in human breast cancer, where the extent of lymph node lymphangiogenesis was correlated with an increased incidence of distant nonsentinel lymph node metastases *(30)*. Further support for the metastasis-enhancing function of lymph node lymphangiogenesis stems from recent studies in experimental models of cutaneous malignant melanomas and nasopharyngeal carcinomas *(31,32)*. Taken together, these studies indicate that the lymphatic vessel expansion within tumor-draining and tumor-affected lymph nodes might represent a new target for inhibiting metastatic cancer spread and, potentially, for the imaging/early detection of lymphatic micrometastases.

4. TUMOR LYMPHANGIOGENESIS AND METASTASIS IN HUMAN CANCERS

When the new concept of tumor-induced lymphangiogenesis—and its promoting effect on cancer metastasis toward lymph nodes—was first identified in mouse models of cancer *(18–21)*, it remained unclear whether these findings were restricted to animal models or whether they might also apply to human cancer spread. One of the most relevant human cancers in this regard is human cutaneous malignant melanoma. Melanomas of the skin can metastasize at an early-tumor stage, and metastasis occurs predominantly via lymphatic vessels to draining sentinel lymph nodes *(33)*. Thus, sentinel lymph node biopsies are routinely performed to evaluate the prognosis of patients with malignant melanomas, and the detection of lymph node metastasis impacts on the staging, the therapy, and the prognosis of the patients *(34,35)*.

In a first study, we compared melanomas that had either metastasized or not, but that were otherwise identical with regard to tumor thickness, location, and patient characteristics. Importantly, we found enhanced tumor lymphangiogenesis in the primary tumors of patients who developed metastases, as compared with patients who did not develop metastases over at least

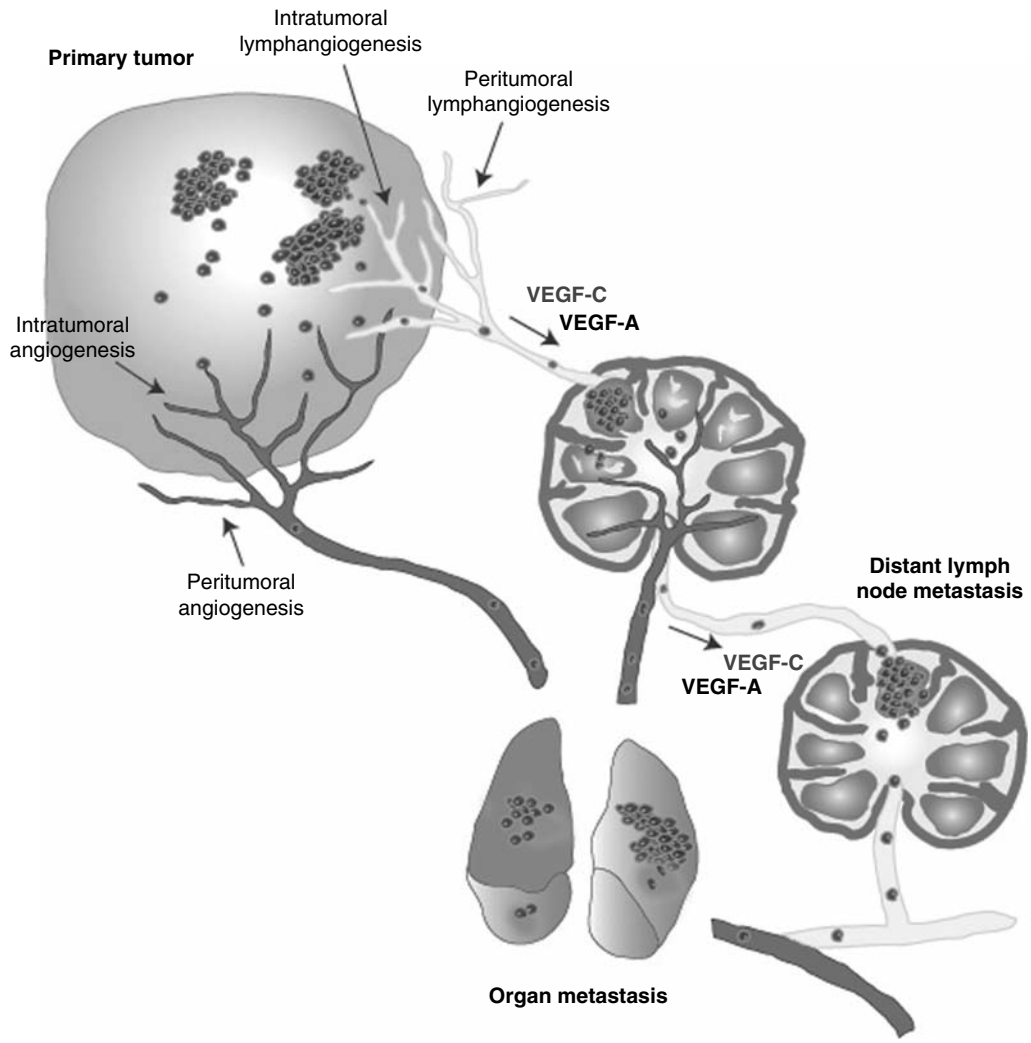

Fig. 1. Tumor and Lymph Node Lymphangiogenesis Promote Cancer Metastasis. In addition to angiogenesis, primary tumors can also induce lymphangiogenesis, often mediated by secretion of VEGF-C or VEGF-A which bind to specific receptors (VEGFR-3 and/or VEGFR-2) on lymphatic endothelium. At the same time, VEGF-A or VEGF-C are drained, via lymphatic vessels, toward the sentinel lymph nodes where they also promote expansion of the lymphatic vessel network. Lymph node lymphangiogenesis facilitates the metastatic seeding by tumor cells which in turn further promote lymphatic vessel growth, thereby promoting metastasis to nonsentinel lymph nodes and, likely, via the thoracic duct to the blood vasculature and distant organs. Modified after *(40,42)*.

3 years after surgery *(36)*. Moreover, an increased level of tumor lymphangiogenesis in the primary tumor was associated with reduced disease-free and overall survival. In a second study, we found that the extent of tumor lymphangiogenesis, as assessed by the relative tissue area covered by lymphatic vessels stained for the lymphatic marker LYVE-1, was the most significant prognostic predictor of the presence of metastasis within the sentinel lymph nodes *(29)*. It is of interest that the prognostic value of tumor lymphangiogenesis was more significant than that of tumor thickness *(29)*.

There have been efforts to identify the nature of the lymphangiogenic activity in human melanomas. Several studies found that the levels of VEGF-C expression were significantly correlated with lymphangiogenesis in primary tumors *(29)* and with lymph node metastasis *(29,37,38)* but more studies in this regard are needed. In contrast, there has not been a correlation of the expression levels of VEGF-D and the incidence of lymph node metastasis *(29,37)*, although VEGF-D has been found in one study to be expressed in melanomas and to have a correlation with tumor angiogenesis *(39)*. It remains to be investigated whether other lymphangiogenic factors, such as hepatocyte growth factor or fibroblast growth factor-2, might also contribute to lymphatic vessel activation in melanoma.

There has been a great interest in investigating the potential contribution of lymphangiogenesis, and of the expression of distinct lymphangiogenic factors, toward cancer metastasis in different human cancer types, in particular in epithelial cancers *(40–42)*. Overall, more than 70 studies have been published regarding the potential influence of VEGF-C expression on cancer metastases, and more than 75% of these studies found a significant positive correlation between VEGF-C expression levels and lymph node metastasis *(40)*. It is of interest that evaluation of VEGF-C expression by immunohistochemistry more often revealed a positive correlation than evaluation by mRNA-based techniques or ELISA *(40)*. In contrast, the association of VEGF-D expression with lymph node metastasis has remained less clear. VEGF-D mRNA levels or protein expression levels were found to be elevated *(43–45)* or decreased in tumor samples *(46–49)* of cancer patients with metastatic tumors.

Overall, these studies reveal VEGF-C as a major lymphangiogenic factor in the majority of human cancers. The divergent results found in some of these clinical investigations may in part be explained by the different methods employed to quantitate lymphangiogenesis and growth factor expression.

5. OUTLOOK

The potential clinical relevance of active tumor lymphangiogenesis has been controversial for a few years after the original discovery of tumor-induced lymphatic vessel expansion. However, the impact of tumor lymphangiogenesis on the promotion of lymph node metastasis has now been documented in dozens of reports—both in experimental animal tumor models and in clinicopathological studies in many different types of human cancers. Thus, tumor-associated lymphatic vessel growth represents a novel prognostic indicator for the risk of cancer metastasis. The new concept of tumor-induced lymph node lymphangiogenesis identifies a new target for the possible prevention, therapy, and possible imaging of cancer metastases. However, there is a need for additional prospective clinical studies in larger cohorts of patients. Although VEGF-C has been found to represent the major lymphangiogenic activity in several types of cancers, the mechanisms of interaction between metastatic cancer cells and lymphatic endothelium—in the primary tumor and within the draining lymph nodes—remain at present poorly characterized. Identification of such molecular and cellular mechanisms will likely lead to additional therapeutic strategies aimed at limiting malignant cancer spread.

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24 T_{reg}, Chemokines, and Other Small Molecules: Role in Metastasis and Its Prevention

Experiences in Melanoma Immunobiology

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Contents

ABSTRACT

It has long been known that malignant tumors contain variable numbers of lymphocytes referred to as tumor-infiltrating lymphocytes (TIL). In melanoma, the intensity of this lymphocytic infiltrate is correlated with outcome measures, though there is some debate in the literature that such an association may only exist for certain melanomas, for example, of a certain thickness. However, early studies on melanoma TIL did not immunophenotype these cells, and recent data have revealed that the composition of the tumoral lymphocytic infiltrate is not homogenous, but rather represents varying contributions from many lymphocytic subsets. Furthermore, the function of CD8 TIL is often compromised as a result of the accumulation of immunoregulatory cells and various tumor escape mechanisms. Nevertheless, the increase in our understanding of immunobiology has facilitated the development of logical immunotherapeutic strategies to overcome such hurdles. Examples of these strategies which are currently

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being tested in clinical trials and which are discussed in-depth include blocking antibodies against CTLA-4 and therapies to deplete Foxp3$^+$ regulatory T cells (T_{reg}), which will hopefully enhance existing immunotherapeutical protocols such as tumor immunization. In addition to strategies designed to tip the balance between antitumor immunity and regulatory T-cell function, a greater understanding of the small molecules which govern the migration of immunocytes and tumor cells (chemokines) has made possible the development of strategies to selectively impede certain immunocyte populations into the tumor microenvironment, as well as to abrogate the chemokine-mediated metastasis of tumor cells. This chapter highlights some of these current developments occurring in the field of immunological cancer therapy, and illustrates how these developments resulted from a greater understanding of basic immunobiology.

Key Words: TIL; melanoma; immunotherapy; CTLA-4; chemokine; immunobiology; metastasis; immunoregulation; T_{reg}; monoclonal antibody; adjuvant

1. INTRODUCTION

Metastatic melanoma responds poorly to most forms of systemic therapy *(1,7)*. Chemotherapy, using either single or multiple agents, induces short-lived responses in 10–40% of patients, but evidence for a significant impact on survival is lacking *(1,7)*. While the individual prognosis of patients with many other malignancies has improved significantly over the last years due to newly acquired therapeutic options *(2–6)*, the prognosis of patients with advanced stage melanoma still remains devastating. For these reasons, researchers are looking at alternative strategies for treating melanoma. Recently, some forms of immunotherapy have been shown to be potentially curative in some patients with metastatic melanoma *(1,7)*, opening a new chapter of research into immunological therapies for melanoma and other malignancies. Some novel immunological treatments and the rationale behind their use in malignant melanoma will be explored in this chapter, with a special emphasis on developments with the anti-CTLA-4 monoclonal antibody (mAb) which is a prototypical immunotherapeutical agent that highlights how our growing understanding of immunobiology can be translated into logical therapeutic strategies.

2. EVOLUTION OF THE TUMOR-INFILTRATING LYMPHOCYTE CONCEPT

More than 100 years ago, malignant tumors were first noted to contain variable numbers of lymphocytes *(8)*, which have come to be known as tumor-infiltrating lymphocytes (TILs). Initially, these TILs were thought to reflect the origin of cancer at sites of chronic inflammation *(8)*, and later it was debated whether TIL provided a favorable environment for cancer growth or were evidence of the host's attempt to eliminate cancer *(9)*. A relationship was first identified between the extent of immune cell infiltration and the prognosis in 1949 in cases of breast cancer *(10)*. In 1969, the lymphocytic infiltration of primary cutaneous melanoma was first described by Clark et al. *(11)*, which Day et al. *(12)* later found to be of prognostic significance. Patients with a moderate-to-marked lymphocytic infiltrate within their primary melanoma had a significantly better prognosis and a three-time higher 5-year survival rate than patients with a sparse or absent lymphocytic infiltrate *(12)*. Elder et al. *(13)* differentiated the lymphocytic infiltrate into brisk, nonbrisk, or absent, according to its intensity, and demonstrated that TILs were of prognostic significance only in vertical growth phase melanoma, but not in radial growth phase melanomas, regardless of whether the melanoma was in situ or invasive *(13,14)*. Also, the amount of TIL in the primary tumor has been found to be inversely correlated with the probability for lymph node metastases *(14,15)*, and of those patients with regional lymph

node metastases, patients having more marked lymphocytic responses in their metastatic melanoma showed significantly higher disease-free survival rates *(12,16)*. However, the results of other studies did not convincingly demonstrate that brisk TIL infiltrates were associated with better patient outcomes *(17–19)*. These discrepant results may in part be explained by differences in patient populations investigated, with particular reference to the thickness of patients' melanomas *(15)*; however, due to technical limitations of the time, these studies did not immunophenotype TIL. Given the explosion of immunological data, and the greater characterization and subclassification of lymphocytes, an effort is being made in new studies to determine whether differences in the composition of TIL impact patient outcomes *(20–23)*. Furthermore, the identification of how immunocytes and tumor cells utilize chemical signals to move throughout the body has initiated new avenues of immunotherapy research, the results of which are eagerly anticipated.

3. IMMUNOPHENOTYPING AND SUBTYPING TIL

Studies on IL-2-cultured TIL demonstrated that their cellular composition varies in individual patients, ranging from 90% $CD4^+$ T cells to 90% $CD8^+$ T cells *(24–26)*, with highly specific cytolytic activity and clinical response correlating with the presence of tumor-specific $CD8^+$ T cells *(15,16,23)*. However despite the presence of tumor-specific cytotoxic CD8 T cells in melanoma lesions, and although most melanoma patients treated with TIL adoptive transfer show a brisk T-cell rich infiltrate, the immune response to melanoma is often inadequate for tumor control, with most patients developing progressive disease resulting in death *(23,27)*. Such a failure of immunity is highlighted by data both from human *(28)* and murine *(29)* tumor models that show that CD8 TILs are generally composed of quiescent and/or functionally anergic effector/memory T cells *(22)*. A myriad of tumor escape mechanisms *(30)*, which are discussed in Chapter 43 and which are briefly summarized in Table 1, likely work together to affect this immune compromise. Since most research has focused upon CD8 T cells, the significance of other immunocyte populations is unclear, despite the presence of a heterogeneous mixture of inflammatory cells within the tumor microenvironment *(21,31–33)*. For example, melanoma-specific CD4 TILs have been demonstrated *(22,34)* and some CD4 TIL can directly lyse tumor cells *(35,36)* and eliminate melanoma in animal models *(20,37)*, an observation that is in keeping with the presentation of some immunogenic melanoma peptides by HLA class II molecules *(26)*. Further subclassification of the CD4 cell compartment has revealed another population of cells, which is variably present within the tumor environment *(32,33,38–40)*. These $CD4^+$ $CD25^+$(high)/$Foxp3^+$ regulatory T cells (T_{reg}) represent 5–10% of human CD4 T cells and function as immunological repressors by inhibiting $CD4^+$, $CD8^+$, and NK-cell responses *(41)*. A deficiency of T_{reg}, either occurring naturally *(42,43)* or induced experimentally *(44,45)*, is associated with massive T-cell lymphoproliferation and multiorgan autoimmunity *(42–45)*, illustrating how a subset of T_{reg} is important for mediating self-tolerance (natural T_{reg}) *(46,47)*. However, it is also apparent that some T_{reg} mediate the response to nonself antigens (induced T_{reg}) *(46,47)*. Interestingly, T_{reg} are significantly increased in patients with epithelial malignancies and T_{reg} TIL have also been shown to be more represented in advanced melanocytic lesions, including metastatic tumors *(32,33)*, and thus the accumulation of these cells may be associated with clonal evolution of melanoma and disease progression *(48)*. Recent data have also shown that T_{reg} are overrepresented in lymph nodes harboring metastatic melanoma *(32)*, which may be related to the ability of cancer cells to liberate substances which are strongly chemoattractive for $T_{reg.}$ *(49,50)*. Furthermore, tumor cells can likely promote the development of T_{reg}, as melanoma-conditioned media was shown to induce the differentiation of T_{reg} in experimental studies *(51)*. It is tempting to speculate that the increased frequency of T_{reg} in advanced malignant lesions *(32,39,40)* may, in part, explain the development of anergy of CD8 TIL

Table 1
Selected Tumor Escape Mechanisms that Mitigate the Antitumor Response

Mechanism	*Proposed basis of tumor escape mechanism*
FasL Expression	Tumor-derived Fas-ligand (FasL, CD178) binds TIL Fas (CD95) to induce apoptosis *(22,136)*.
RCAS1 Expression	Tumor-derived RCAS1, expressed on a minority of melanoma cells, acts as a ligand for a putative activation-associated lymphocyte receptor that inhibits growth and induces apoptosis of TIL. RCAS1 expression may also skew T cells toward T_H2 differentiation through secretion of IL-10/TGF-β *(22,137–139)*.
Tumor expression of B7-H1	Engagement of the lymphocytic programmed death 1 molecule (PD1) by tumor-associated B7-H1 results in exhaustion/apoptosis of tumor-specific TIL *(22,30,140)*.
High tumor cell burden	Chronic exposure to tumor Ag, particularly without costimulatory signaling, leads to exhaustion/deletion of T cells *(22)*, in part, due to defective T-cell and NK-cell signaling by reduced expression/loss of CD3-ζ chains *(141)* and associated signaling molecules (Zap-70, $p56^{lck}$) *(141,142)*. Exhaustion may also be manifested by dysfunctional IL-2 signaling caused by reduced Jak3 expression *(143)*. Chronic exposure to tumor antigen can also lead to activation-induced cell death (AICD) *(22)*.
Release of ROS	Macrophages and melanoma cells are both sources of reactive oxygen species (ROS) *(144)*. Although melanoma is relatively resistant to ROS because of an extensive antioxidant network *(144)*, ROS may contribute to the loss of the aforementioned signaling molecules in TIL (i.e., CD3-ζ) *(22)*.
Tumor expression of CCL22/CCL17	Tumor-derived CCL22 and CCL17 chemoattract T_{reg} TIL that downregulate antitumor immunity by cell-to-cell or cytokine-mediated suppression *(22,49)*.
Tumor expression of IDO	Tumor expression of indolamine 2,3-dioxygenase (IDO), an enzyme which catalyzes tryptophan degradation, results in an impairment in the accumulation of tumor-specific T cells *(145)*. IDO seems to block proliferation of T cells, which are extremely sensitive to tryptophan shortage *(145)*.
Generation of epitope loss variants	Because some melanoma Ag are not important for tumor cell survival (melanosomal proteins), loss of tumor-associated antigens (TAAs) occurs in 5–20% of patients with metastatic melanoma *(30)*. These clones can be selected for by immunotherapy protocols targeting a single TAA.
Tumor HLA Ag loss	Loss of HLA class I Ags can occur selectively, or more generally due to mutations/deletion of β2-microglobulin genes *(30)*, as β2-microglobulin stabilizes HLA class I molecules *(82)*. This loss abrogates tumor recognition by Ag-specific T cells *(30,146)*.

(*Continued*)

Table 1
(Continued)

Mechanism	*Proposed basis of tumor escape mechanism*
Tumor expression of certain HLA class Ib molecules	The HLA class Ib molecules, HLA-E and HLA-G, protect from NK cell destruction by masking the loss of HLA class I molecules (see "missing self" hypothesis of NK cell-mediated lysis) *(22,147,148)*. HLA-E presents a peptide derived from the membrane localization signal of HLA-G, maintaining expression of "self" without presenting immunogenic peptides *(147)*. This also serves to inhibit effector/memory T-cell function, as activated T cells often upregulate inhibitory NK receptors *(22)*.
Tumor expression of MMPs	Tumor-derived matrix metalloproteinases (MMPs) may cleave the high-affinity IL-2 receptor (CD25, IL-2Rα), abrogating the activation and subsequent proliferation of tumor-specific T cells *(22,149)*.
Impairment of peptide processing	Downregulated expression of transporter-associated with antigen processing (TAP) and immunoproteasome components, such as LMP-2 and LMP-7, sequester or abrogate the production of immunogenic peptides *(30,150)*.
Tumor expression of CEACAM1	Carcinoembryonic Ag cell adhesion molecule 1 (CEACAM1) is broadly expressed by immunocytes and by melanoma, especially those melanomas associated with a bad prognosis *(151)*. CEACAM1 homophilic interactions inhibit TIL effector functions *(151)*.
Mitigation of proinflammatory signals	The constitutive activity of the signal transducer and activator of transcription 3 molecule (Stat3) in some melanoma cell lines inhibits the production of proinflammatory danger signals, which are important for the functional maturation of dendritic cells (DCs) *(30,152)*, which thereby may lead to the tolerization of TIL.

(52,53), a premise which is supported by various model systems where increased T_{reg} TIL are associated with poor CTL responses *(54)*, and the depletion of T_{reg} evokes effective antitumor immunity *(41,47)*. Given the abundant experimental data and the findings in other malignancies that suggest that increased T_{reg} TIL are associated with a worse prognosis, worse tumor control, and weaker CTL responses *(33,55,56)*, studies are ongoing to investigate whether a correlation exists between the prognosis in melanoma and the frequency of T_{reg} TIL. Similarly, further study will be required to garner additional information on other lymphocyte subsets to determine whether their presence or absence has any implication for melanoma management.

4. STRATEGIES TO AUGMENT ANTIMELANOMA IMMUNITY

A number of different strategies are being pursued to enhance the frequency of melanoma specific T cells and thereby alter the balance between tumor-specific immunity and immune regulatory processes. The collection and in vitro expansion of TIL followed by their adoptive transfer have resulted in successful tumor eradication in murine models *(57)* and has been utilized with limited success in human studies *(58,59)*. Other methods include the vaccination of patients with melanoma antigen or the augmentation of natural Ag presentation in vivo

through the use of various adjuvants *(60–62)*. Steps taken to enhance natural antigen presentation have included the administration of a number of cytokines. IL-2, perhaps the most important of lymphocyte growth factors, has been intensely investigated for the therapy of melanoma *(24,63)*, and the finding that IL-2 is potentially curative for a small number of patients with malignant melanoma *(63)* has led to its FDA approval for this purpose *(1)*. Interferon-alpha (IFN-α) *(64)*, IL-12 *(65,66)*, and GM-CSF *(67,68)* have also been used as tumor adjuvants to enhance antimelanoma immunity with some success. Interestingly, the use of GM-CSF resulted in accumulations of large numbers of professional antigen-presenting cells *(68,69)* and, as a result, has led to its integration into a promising vaccination protocol which employs irradiated autologous tumor cells that have been genetically engineered to produce large amounts of this factor *(70)*. Various other vaccine strategies have been attempted, including the use of tumor-specific peptides, especially those derived from melanosomal Ag, with or without dendritic cells (DCs) *(61,62,71–76)*. Although some of the results from the aforementioned protocols have shown promise in the treatment of melanoma, with some studies demonstrating significant tumor rejection *(61)*, on the whole, the results have not been overwhelmingly successful *(60,73,75)*. Various biologic therapies with potentially immune modulating function are being developed for the management of malignancy *(77)*, and it is hoped these agents will improve the prognosis of patients with advanced melanoma.

5. ANTI-CTLA-4 mAb USE IN MELANOMA IMMUNOTHERAPY

A novel strategy to increase the frequency of tumor-specific T cells is the use of mAb against critical regulators of lymphocyte activation and proliferation *(62)*. One such molecule is the cytotoxic T-cell lymphocyte antigen-4 (CTLA-4), which is not expressed on naïve T cells, but is upregulated by T cells approximately 3 days following activation *(78,79)*. CTLA-4 is critically important for the contraction of immune responses that is necessary to ensure that other T-cell clonotypes are not dangerously diluted by unopposed clonal expansions *(80)*. CTLA-4 is a high-affinity receptor for B7 ligands *(81)*, which are expressed on mature APCs *(82,83)*. These ligands are critical for delivering the "costimulatory" signal or "signal 2," which is transduced through CD28 and related cell surface molecules *(82,83)*, upon initial Ag encounter and which is necessary for the induction of T-cell activation and proliferation *(80,82)*. CTLA-4 is believed to antagonize T-cell activation/expansion by at least two possible mechanisms. The first involves CTLA-4's 100- to 2,000-fold greater affinity for B7 ligands relative to CD28 *(77,80,81)*, which effectively eliminates costimulatory signally by the sequestration of B7 ligands *(77,81)*. The second mechanism is CTLA-4's recruitment of an inhibitory phosphatase (SHP-2) *(81)*, leading to the extinguishment of downstream T cell receptor (TCR) signaling *(84,85)*. The synergistic effect of these two processes is to halt further expansion of Ag-specific T cells and enhance the attrition of the expanded clonal population, probably by depriving T cells of survival signals which they obtain through low-level TCR signaling *(86)*. Thus, the blockade of CTLA-4 was proposed as an adjuvant to prolong the clonal expansion phase during natural tumor Ag presentation or following tumor vaccination in order to increase the frequency of tumor-reactive T cells *(87)*. Various animal models have validated the effectiveness of CTLA-4 blockade in increasing the clone size of Ag-specific T cells when used in association with tumor vaccination, which was found to be associated with better tumor control in these model systems *(88,89)*. Based on these experimental findings, two humanized anti-CTLA-4 mAb have been developed, which are in advanced clinical trials for the treatment of a variety of malignancies *(90–95)*. The preliminary data from these early trials have been encouraging, with anti-CTLA-4 used alone or in association with tumor vaccination, resulting in better tumor control *(87,90–95)* in some recipients (Fig. 1) *(96)*. However, despite these encouraging results, early trials of CTLA-4

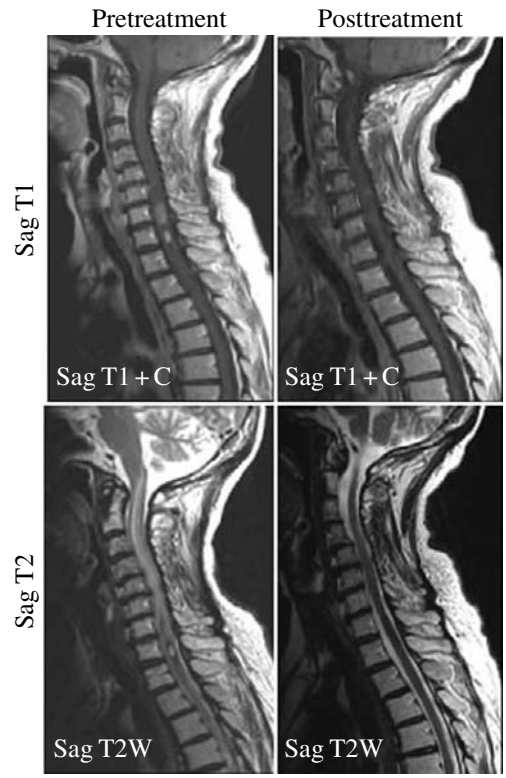

Fig. 1. CTLA-4 blockade results in significant tumor regression in some patients. MRI images of the cervicothoracic spine from a patient with metastatic malignant melanoma which reveal enhancing intraspinal metastases with extensive cord edema prior to treatment with CTLA-4 blockade. Posttherapy images demonstrate complete resolution of the metastases and the accompanying edema. (adapted from Hodi et al. *[96]*).

blockade have been complicated by the development of adverse effects of seemingly autoimmune nature, of which panenteritis (Fig. 2) was the most commonly encountered *(97)*. These adverse effects were found to be variably severe, dose-related, self-limited, and, interestingly, positively correlated with the antitumor effect *(90–95,97–101)*. Given the autoimmune nature of these complications, their emergence may reflect the uncovering of latent self-reactivity, the induction of which may be essential for the effective recognition of nonmutated tumor antigens. While it is tempting to speculate that CTLA-4 blockade may lower the activation threshold of lymphocytes with low-affinity receptors for self-antigens or tumor antigens, it is unlikely that this is the direct mechanism of CTLA-4 blockade, as this receptor is not present at the cell surface at the time of initial antigen encounter *(78,79)*. Rather, it is more likely that prolonged T-cell expansion liberates excessive inflammatory cytokines *(102)*, which are responsible for the adverse effects. Such a "cytokine storm" may work in a bystander fashion to lower the activation threshold of self-specific cells with low-affinity TCR *(103)*, and/or reactivate higher affinity self-specific cells which have been anergized (inactivated) by the constant exposure to self-Ag *(81)*. The findings that CTLA-4 blockade, used together with high-dose IL-2 therapy, resulted in a more serious side-effect profile *(63,104,105)*, including the risk of bowel perforation *(105)*, is in keeping with this hypothesized mechanism as it is likely that the introduction of exogenous cytokine further exacerbates the "cytokine storm" induced by CTLA-4 blockade. This hypothesis also satisfactorily explains how the antitumor effect of anti-CTLA-4 mAb, resulting from the "therapeutic lymphoproliferation," can be dissociated from the cytokine-driven, adverse events *(101)*.

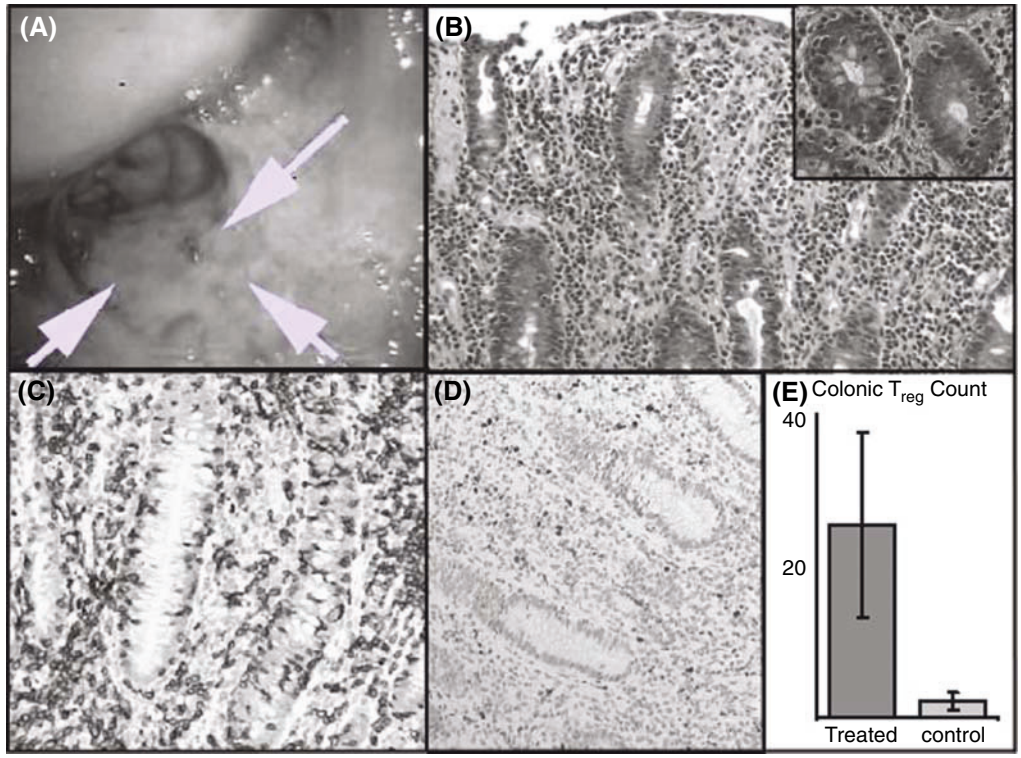

Fig. 2. Patients undergoing CTLA-4 blockade develop autoimmune-like adverse effects, including a panenteritis-resembling autoimmune enteritis (AIE) *(97)*. (**A**) A representative colonoscopic image demonstrating a large area of ulceration in the rectum. (**B**) A representative photomicrograph of H&E-stained colonic epithelium demonstrating a variably dense lymphoplasmacytic expansion of the lamina propria, and an increased number of intraepithelial lymphocytes accompanied by a marked increase in apoptotic enterocytes (10×, inset – 40×). (**C**) A representative photomicrograph of a CD3-immunostained colonic section demonstrating a large increase in lamina propria and intraepithelial T cells (40×). (**D**) A representative photomicrograph of a Foxp3 immunostained section of colon demonstrating a preserved population of $Foxp3^+$ T_{reg} in the inflammatory infiltrate (10×). (**E**) A graphical representation of colonic $Foxp3^+$ T_{reg} enumeration in control patients and patients treated with CTLA-4 blockade. (adapted from Oble et al. *[97]*).

Another plausible explanation for the autoimmune sequelae, and possibly the antitumor effect of CTLA-4 blockade, is the abrogation of T_{reg} function. CTLA-4 is expressed constitutively by T_{reg} *(106)*, and the pattern of autoimmune disease in anti-CTLA-4 mAb-treated patients is strikingly similar to that in patients with immune dysregulation, polyendocrinopathy, enteropathy, and X-linkage (IPEX) syndrome *(107)*, who are naturally deficient of functional T_{reg} as a result of mutations in Foxp3 *(43,107)*. Furthermore, IPEX patients and Foxp3-deficient mice have a phenotype similar to that of CTLA-4-deficient mice *(108,109)*, suggesting a critical role for CTLA-4 in T_{reg} function. Though, somewhat surprisingly, T_{reg} are present in anti-CTLA-4 mAb-treated patients (Fig. 2) *(97)*, suggesting that CTLA-4 blockade may disrupt the function of T_{reg} without mediating their depletion *(97)*—an observation also made in experimental models *(110,111)*. However, the recent finding, in a small series, that an inverse relationship exists between the frequency of T_{reg} in metastatic melanoma and both the extent of necrosis and the frequency of cytotoxic T cells in such lesions (Fig. 3) *(101)*, argues that T_{reg} in anti-CTLA-4-treated patients (at least at the doses utilized in this study) still retain some activity and limit the cytotoxic response to tumor antigen *(101)*. Therefore, patients treated with CTLA-4 blockade may still benefit from

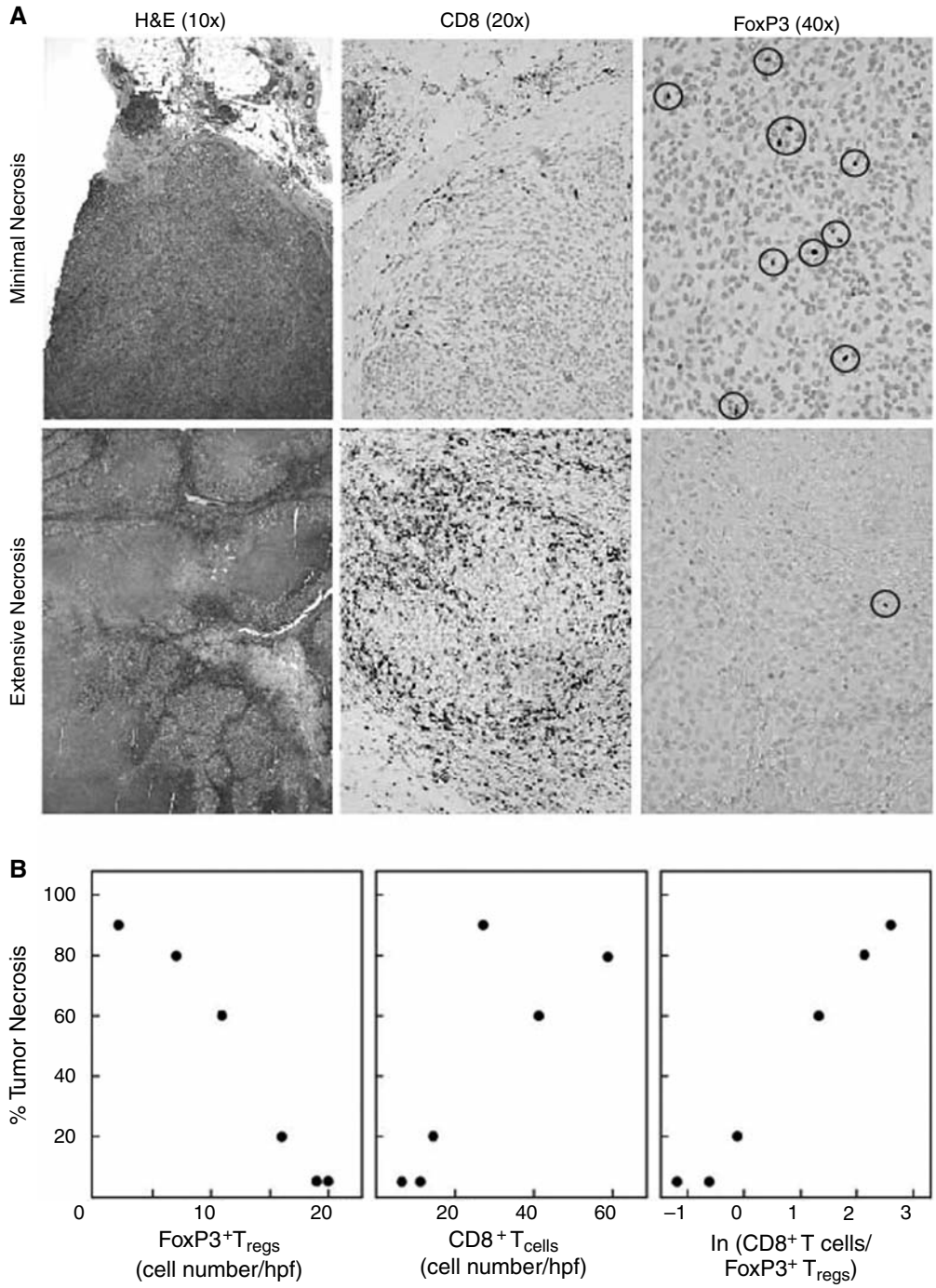

Fig. 3. The ratio of tumor infiltrating CD8 T cells to Foxp3$^+$ T_{reg} TIL following anti-CTLA-4 treatment is tightly correlated with the extent of tumor necrosis. (**A**) Representative photomicrographs demonstrating CD8 and Foxp3$^+$ T_{reg} TIL in melanoma metastasis exhibiting minimal (*top*) and extensive (*bottom*) necrosis. (**B**) Graphical demonstration of the relationship between Foxp3$^+$ T_{reg} TIL, CD8 TIL, and tumor necrosis. (adapted from Hodi et al. *[101]*).

further measures to deplete T_{reg}. Though the mechanism of CTLA-4 blockade is not clear, the results of early trials have indicated that such an approach may be a promising strategy to enhance the immune response against melanoma, which illustrates how successful therapeutic strategies can be translated directly from a greater understanding of immunobiology.

6. T_{REG} DEPLETION STRATEGIES IN THE IMMUNOTHERAPY OF MELANOMA

A number of different strategies are being pursued to deplete T_{reg} and thereby enhance anti-melanoma immunity by mitigating immune regulatory processes. One such strategy is the depletion and/or disruption of T_{reg} using an mAb directed against the IL-2 receptor alpha chain (IL-2Rα, CD25) which, similar to CTLA-4, is not found on naïve T cells but is expressed constitutively by T_{reg} *(82,112)*. In experimental studies, antibodies against CD25, or fusion proteins consisting of anti-CD25 mAb coupled to various toxins, have been shown to abolish T_{reg} function without depleting these cells *(113)*. However, despite the success of anti-CD25 therapy at inducing major immune responses in phase I clinical trials *(114)*, this therapy did not result in any objective responses even though T_{reg} cell numbers were reduced in vivo *(115)*. A related strategy targeting CD25 is the use of the denileukin diftitox (ONTAK), a recombinant cytotoxic protein composed of portions of the diphtheria toxin together with human IL-2, that binds to CD25 and, after internalization, leads to cell death *(55,116,117)*. Despite being effective in clinical trials for other malignancies *(116)*, the use of this experimental agent for the treatment of melanoma was not associated with a reduction in circulating T_{reg} or tumor regression in patients with metastatic melanoma *(117)*. It is unclear why these therapies have been less efficacious than anticipated; however, one explanation may be the unwanted depletion of tumor-specific T cells, since the upregulation of CD25 and the expression of IL-2 are some of the earliest events in T-cell activation *(82,118)*. Therefore, the tumor-specific population of cells one hopes to expand through natural antigen presentation or tumor Ag vaccination may be depleted by these CD25-directed agents. Emerging data will clarify whether a role exists for CD25-targeted therapy in the management of cancer.

An alternative strategy to abrogate the function of T_{reg} involves interrupting the migration of these regulatory cells into the tumor microenvironment by utilizing mAb directed against chemokines and their receptors. Chemokines are small polypeptide molecules that interact with their cognate chemokine receptors, present on the surface of different cells *(9)*. These interactions are responsible for directing cells to specific sites, a process called homing *(9)*. In general, cells express a characteristic repertoire of chemokine receptors and migrate concentration dependently toward their respective chemokine ligands. The majority of T_{reg} express high levels of the chemokine receptors CCR4 (the receptor for CCL22) *(50,119)* and CCR6 (the receptor for CCL20) *(120)* which are targets for T_{reg}-modulating mAbs, a strategy which has proven to be effective in experimental models where, for example, an anti-CCL22 mAb reduced T_{reg} migration to ovarian tumors *(121)*. Accordingly, it is anticipated that ongoing clinical trials using an mAb against CCR4 for the treatment of hematological malignancies *(122)* will be expanded to include patients with various other malignancies, of which melanoma would be an ideal candidate. Although a number of different strategies are being pursued to deplete T_{reg}, the potential to induce major autoimmune adverse effects will necessitate much additional research to optimize T_{reg}-depleting techniques.

7. ADDITIONAL CHEMOKINE-TARGETED IMMUNOTHERAPY STRATEGIES

Chemokines also play a role in tumor metastasis independent of their role in attracting T_{reg} to the tumor microenvironment, as highlighted in Chapter 26. For example, common sites of distant melanoma metastasis share a similar profile of chemokine expression (CXCL12/SDF-1α, CCL21/

6Ckine, and CCL27/CTACK) *(123,124)*, while melanoma cells express the corresponding chemokine receptors (CXCR4, CCR7, and CCR10) at high levels *(123)*. CXCR4 is an especially attractive target for immunotherapy since, although it is overexpressed in melanoma *(123)*, it is not expressed or only minimally expressed by normal tissues *(124)*, and models have shown that CXCR4 neutralization delays tumor growth and suppresses lymph node metastasis *(125,126)*. Consequently, antagonism of the chemokine receptors CCR7 and CXCR4 by mAb and small-molecule inhibitors is currently being investigated in clinical trials for the treatment of malignant melanoma *(124,127–129)*. Other strategies being tested for the modulation of chemokine expression involve techniques employing genetic manipulation *(130–132)* and therapies that target the nuclear factor-kappa B (NF-κB) pathway *(133,134)*, since NF-κB activation upregulates the transcription of many chemokines *(135)*. The preliminary data from the use of these novel immunomodulating agents have been, in some cases, encouraging thus far *(132)*, and the results of these clinical trials are eagerly anticipated so that successful strategies can be integrated into routine melanoma management.

8. CONCLUDING REMARKS

Since the prognostic significance of TIL was discovered, much has been learned about the immunobiology of lymphocytes and the small molecules that govern the behavior of these cells. Advances on T-cell activation, tolerance induction, and the identification of new immunocyte subsets have illustrated that the extent of antimelanoma immunity is more complex than initially thought, and it is likely that the composition of TIL may prove to be as important as the "briskness" of the lymphocytic infiltrate. However, much is still to be learned about the interactions of TIL subsets, both with one another and with tumor cells, as well as how novel biological therapies influence TIL behavior. This additional insight will surely contribute to more successful immunotherapy for melanoma and other malignancies.

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VII Molecular Targeted Therapy Against Growth Factor Receptors, Signaling Pathways and Angiogenesis as Therapeutic Targets

25 Molecular Targeting of Lymphangiogenesis and Tumor Metastasis

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Contents

ABSTRACT

Recent discoveries of essential factors regulating the development of the lymphatic system have resulted in the establishment of new biological models and the identification of lymphatic-specific markers, which have enabled further understanding of the lymphatic system and its associated diseases. Critical factors for development of the lymphatic system include vascular endothelial growth factor-C (VEGF-C) and its receptor VEGFR-3. In many cancers, expression of the VEGFR-3 ligands, VEGF-C and VEGF-D, is correlated with tumor metastasis. A number of additional molecules have since been identified to promote lymphangiogenesis as well as

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metastasis, including a number of receptor tyrosine kinases and downstream signaling molecules, chemokines and chemokine receptors, matrix and basement membrane proteins, and inflammatory mediators such as COX-2. The development of therapeutics interfering with lymphangiogenesis, particularly those inhibiting VEGFR-3 signaling, has therefore been of growing interest in oncology. Among these therapeutics are tyrosine kinase inhibitors, blocking antibodies, and ligand traps. As adjuvant therapy, these inhibitors offer great potential for impeding disease progression.

Key Words: Lymphangiogenesis; lymphatic metastasis; lymphangiogenic molecules; tyrosine kinase inhibitors; soluble receptors; blocking antibodies

1. INTRODUCTION

Sustained primary tumor growth is dependent on a continuous supply of oxygen and nutrients provided by tumor-associated blood vessels formed through the process of angiogenesis *(1)*. Cancer dissemination, however, is dependent on both the tumor-associated blood and lymphatic vasculature *(2)*. Lymphangiogenesis, defined as the growth of new lymphatic vessels, is especially important for metastasis, as newly formed lymphatic capillaries associated with the primary tumor provide a route for tumor cells to disseminate into regional lymph nodes and to other more distant organs. While metastasis to other organs is the major cause of cancer patient mortality, it is metastasis to the lymph nodes that serves as a crucial prognostic indicator. Since lymphangiogenesis is critical for tumor cell dissemination into lymphatic vessels and lymph nodes, many research efforts have focused on identifying lymphangiogenic molecules and evaluating the efficiency and use of lymphangiogenesis inhibitors as therapeutic agents. Recently, success has been gained in treating cancer patients by administration of tyrosine kinase inhibitors affecting lymphangiogenic signaling pathways, and even more evidence for the efficacy of adjunct therapy using specific antibodies targeting lymphangiogenic molecules has been gathered from several preclinical in vivo studies indicating a reduction in both primary tumor and metastatic burden. Several ongoing clinical trials conducted with a variety of cancer diseases continue to evaluate the antitumor effects of small molecules that inhibit the signaling pathways important for lymphangiogenesis, including not only those associated with VEGFR-3 activity but other molecules as well.

2. LYMPHANGIOGENIC VEGF RECEPTORS AND LIGANDS

The neoformation of lymphatic capillaries is induced and maintained by several different growth factors and their receptors. The most important of the lymphangiogenic factors are members of the vascular endothelial growth factor receptor (VEGFR) family, namely VEGF-C and VEGF-D, which act through their receptor VEGFR-3 *(3,4)*. During the early stages of development, VEGFR-3 is expressed on all vessels, but later becomes restricted to developing lymphatic vessels *(5)*. Postnatally, VEGFR-3 expression is largely confined to the lymphatic endothelium, but becomes upregulated during physiological processes accompanied by neovascularization. VEGFR-3 expression is also induced during pathological angiogenesis and lymphangiogenesis with VEGFR-3 found on tumor-associated blood and lymphatic capillaries *(6)*. While the immature unprocessed forms of VEGF-C and VEGF-D interact primarily with VEGFR-3, proteolytic processing of these molecules enhances their affinity for VEGFR-3 with the fully cleaved forms also capable of binding to and activating VEGFR-2 *(4,7–9)*. While VEGFR-2 is expressed mainly in arterial endothelial cells and promotes the growth of new blood vessels, this receptor is also found on lymphatic endothelial cells (LECs) and

influences lymphatic induction mediated by the mature forms of VEGF-C and VEGF-D or by the VEGFR-2 ligand VEGF-A *(10,11)*. Furthermore, heterodimerization of VEGFR-2 and VEGFR-3 can also mediate signals required for lymphangiogenesis *(12)*.

In addition to the VEGF receptors, VEGF ligands can also bind to neuropilins. Neuropilin-1 and neuropilin-2 (NP1 and NP2) were originally identified as receptors for class III semaphorins, which mediate axonal guidance and retraction *(13,14)*. Neuropilins are transmembrane non-tyrosine kinase glycoproteins that have a short cytoplasmic domain with limited signaling capability. To mediate semaphorin-induced signals, neuropilins associate with plexins, which act as the signal transducers, while VEGF signals are transduced by associated VEGF receptors *(15,16)*. In the vascular system NP1 is expressed in arteries, while NP2 is expressed in the lymphatic system and at low levels in veins *(17,18)*. Although mice deficient for NP2 are viable, they display mild neuronal defects and lack lymphatic capillaries and small lymphatic vessels at birth, while arteries, veins, and larger lymphatic vessels remain intact *(19)*. The lymphatic defect in these mice is likely due to the ability of NP2 to bind the lymphangiogenic growth factors VEGF-C and VEGF-D and associate with VEGFR-3 *(20)*.

3. ANGIOPOIETINS AND TIE RECEPTORS

Angiopoietins are a family of vascular growth factors with at least four known members, all of which bind to the Tie2 receptor *(21,22)*. Of these, angiopoietin-1 (Ang-1) and the mouse/human interspecies ortholog Ang-3/Ang-4 are mainly agonists of the receptor *(21,22)*, while the role of Ang-2 is more controversial and is considered a context-dependent antagonist or weak agonist *(23–25)*. In the blood vascular system, Tie2 and Ang-1 are required for developmental angiogenesis, vessel remodeling, integrity, permeability, and stability *(26,27)*, while Ang-2 is required for postnatal angiogenesis and vessel remodeling *(28)*. In the lymphatic system, both Ang-1 and Ang-2 have been shown to function mainly as agonists with Ang-2 null mice displaying a defective lymphatic microstructure and deranged smooth muscle cell association with the lymphatic endothelium at the site of the collecting lymphatics *(28)*. While Ang-1 can rescue the lymphatic defect in Ang-2 mice, it is unable to compensate for the Ang-2 angiogenic defect *(28)*. Furthermore, Ang-1 has been shown to promote the growth of new lymphatic vessels *(29,30)*. Tie1 may also play a role in lymphatic vessel development and function as Tie1 null mouse embryos displayed severe edema *(31)*. While Ang-1 and Ang-4 are able to activate Tie1 *(32)*, the ability of Tie1 and Tie2 to heterodimerize may be important for modulation of signaling through these receptors *(33,34)*. The increased expression of Ang-1 has been implicated to promote tumor-associated angiogenesis in a murine cancer model *(35)*. Ang-2 may also stimulate metastasis, perharps by by interacting with the adhesion molecule $\alpha 5\ \beta 1$-integrin mediated pathway and contributing to the acquisition of an invasive phenotype *(36)*. Together, these results imply a crucial role for angiopoietins and Tie receptors in lymphangiogenesis and the metastatic process.

4. CHEMOKINES, CHEMOKINE RECEPTORS, AND OTHER CELL-HOMING MOLECULES

A number of chemokines and chemokine receptors have been implicated in lymphatic metastasis *(37,38)*. While chemokines produced by lymph nodes typically provide a homing signal to immune cells expressing the corresponding receptor, a number of tumor cells express these receptors as well. For example, tumor cells expressing the chemokine receptors CXCR4 or CCR7 can home to lymph nodes, which express high levels of the respective chemokines, namely

CXCL12 and the secondary lymphoid chemokine SLC/CCL21 *(39)*. LECs also secrete CCL21, which acts to attract CCR7-positive tumor cells to lymphatic endothelium thereby permitting access to lymphatic vessels *(40)*. Other molecules involved in leukocyte trafficking and which have been implicated in lymphatic tumor cell dissemination include the cell adhesion molecule CD44 expressed on lymphocytes and certain tumor cells. Its ortholog lymphatic endothelial hyaluronan receptor LYVE-1 is a marker for lymphatic capillaries and macrophages *(41)*.

5. MATRIX AND BASEMENT MEMBRANE–CELL INTERACTION PROTEINS

Factors affecting lymphangiogenesis can also be found in the tumor microenvironment. Matrix proteins such as fibronectin and collagen can modulate VEGFR-3 activity by interacting with β1-integrin *(42)*. Cells expressing VEGFR-3 and stimulated with fibronectin or collagen display increased VEGFR-3 phosphorylation in the absence of VEGF-C or VEGF-D. This activity is mediated by the interaction of integrin β1 with VEGFR-3. Studies of integrin α9β1 have shown that mice lacking this integrin die shortly after birth due to bilateral chylothorax *(43)*. Heparan sulfates, also found in the matrix, may modulate VEGFR-3 activity by increasing the affinity of VEGF-C and VEGF-D for their receptors *(20,44,45)*. In addition, metalloproteases are required for extracellular matrix remodeling during physiological lymphangiogenesis. Both LECs and tumor cells from lymph node metastases have been shown to express a variety of matrix metalloproteases *(46,47)*, which have crucial roles in the dissemination process *(48)*.

6. OTHER MEDIATORS OF LYMPHANGIOGENESIS AND LYMPHATIC METASTASIS

Expression profiling of lymph node metastases in comparison with primary tumors has shown a number of genes differentially regulated *(49, 50)*. While also gene expression analyses of LECs have revealed a number of novel potential mediators of lymphangiogenesis *(51, 52)*, the role of many of these factors in lymphatic development and lymphatic metastasis remains to be determined. Recent investigations, however, have implicated other mediators of lymphangiogenesis and lymphatic metastasis including a number of tyrosine kinase receptors. While VEGF-C/VEGFR-3 signaling is crucial for the initial sprouting of the first Prox-1-positive LECs from the jugular vein and formation of the lymphatic vasculature *(53)*, investigations have shown that the transmembrane ligand ephrinB2, which signals through its tyrosine kinase Eph receptors, is required for proper postnatal lymphangiogenic remodeling *(54)*. Transcriptional profiling revealed that the tyrosine kinase hepatocyte growth factor receptor (HGFR/c-Met) is highly expressed in LECs compared with blood endothelial cells (BECs), and the scatter factor HGF stimulates LEC proliferation, migration, and tube formation *(55)*. In vivo, while HGFR is not expressed on the normal lymphatic endothelium, it is apparently upregulated on regenerating lymphatic endothelium during tissue repair and during inflammation, while HGF stimulates new lymphatic vessel growth acting through a mechanism independent of VEGFR-3 signaling *(55,56)*. In addition to its ability to induce angiogenesis, the fibroblast growth factor FGF-2 induced lymphangiogenesis in the mouse cornea by upregulating expression of VEGF-C *(57,58)*. The platelet-derived growth factor PDGF-BB was also reported to stimulate new lymphatic vessel growth and fibrosarcoma cells expressing PDGF-BB-induced tumor lymphangiogenesis and promoted lymph node metastasis *(59)*. In addition, the insulin growth factors IGF-1 and IGF-2, which act through their tyrosine kinase receptors IGF-1R and IGF-2R, can also stimulate lymphangiogenesis possibly through a direct mechanism independent of VEGFR-3 signaling

(60). Interestingly, stimulation of any of these tyrosine kinase receptors, as well as those of the VEGF and Tie receptor families, results in the activation of similar signaling pathways, which include the phosphatidylinositol 3-kinase (PI3-K), MAP kinases, and Akt *(2)*. Recently, mutational targeting of the PI3-K gene encoding the p110alpha isoform to prevent protein interaction with Ras resulted in a lymphatic defect indicating the importance of this signaling component for proper lymphatic development *(61)*. Cyclooxygenase-2 (COX-2) expression was reported to correlate with VEGF-C expression in lymph node metastases with COX-2 upregulating VEGF-C via the prostaglandin EP_1 receptor and HER-2/Neu tyrosine kinase receptor *(62)*. Podoplanin, a transmembrane glycoprotein, is expressed primarily on the lymphatic endothelium and is required for proper lymphatic vessel development *(63)*. Although the physiological function of podoplanin is unknown, podoplanin becomes upregulated on the invasive front of many carcinomas and promotes tumor cell invasion in the absence of epithelial–mesenchymal transition *(64)*.

7. LYMPHANGIOGENESIS AND TUMOR METASTASIS

Tumor metastasis, the process of tumor cell dissemination to regional lymph nodes and distant organs, is one of the hallmarks of cancer and often the most important cause of morbidity and mortality in cancer patients. Tumor cells can relocate via lymphatic vessels to local lymph nodes by means of local invasion or primarily or secondarily through hematogenous routes. The process of lymphatic metastasis requires multiple changes in the breakaway cell and continuous interactive changes between tumor cells and the surrounding stroma, such as detachment of tumor cells from one another, attachment to the basement membrane, degradation of extracellular stroma, invasion in the lymphatics, and subsequently migration of tumor cells *(65,66)*. Once in the lymphatics, tumor cells are transported by the lymph flow to lymph nodes. When tumor cells to establish in regional and distant lymph nodes, they induce neovascularization, lymphangiogenesis, and sinusoidal hyperplasia (Fig. 1). This also facilitates further relocation to distant organs and evasion from immunologically mediated degradation. Further spread can occur through efferent lymphatic vessels that drain to veins or directly by blood vessels. In addition, disseminated cells can extravasate from lymphatics, and invade to form a deposit in the new location *(65,66)*.

Metastasis is a highly interactive, complex process involving, among other changes, the upregulation of several genes including vascular growth factors and other lymphangiogenic molecules such as those described here *(67)*. Cytokines and chemokines secreted both within primary tumors and at metastatic sites contribute to enhancing the spread of tumor cells into lymph nodes and beyond *(68)*. Among these inducers, the VEGFs, VEGF-C, and VEGF-D and their receptor VEGFR-3, are the most well-established mediators of tumoral lymphangiogenesis and lymphatic metastasis *(69,70)*. A number of reports have indicated that the extent of VEGF-C expression within the primary tumor correlates with the degree of lymphatic dissemination of tumor cells *(71,72)*. In addition to being secreted by the tumor cells themselves, inflammatory cells, for example macrophages, may contribute to the induction of lymphangiogenesis by also producing VEGF-C *(73,74)*. Although not as commonly expressed in tumors as VEGF-C, VEGF-D expression is elevated in certain tumor types and also contributes to lymphatic metastasis *(75)*. Furthermore, VEGF-D is likely important for the metastatic spread via lymphatics of the abnormal smooth muscle cells found in patients suffering from lymphangioleiomyomatosis *(76)*.

Recent results have shown an inhibition of cancer progression by antiangiogenic gene therapy targeting VEGF-triggered pathways if used as a strategy for molecular therapy in certain types of neoplasia in selected patients *(77)*. Since the approval of bevacizumab, a monoclonal antibody

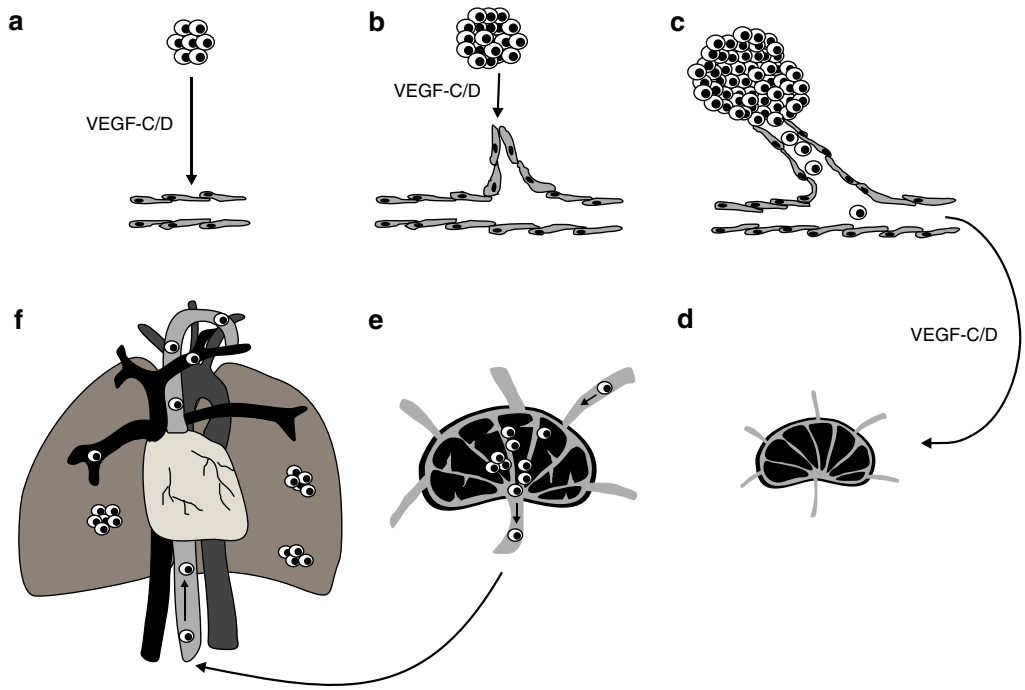

Fig. 1. VEGF-C and VEGF-D Induce Lymphangiogenesis and Promote Lymphatic Tumor Metastasis. (**a**) Tumor cells and tumor stromal cells secrete lymphangiogenic growth factors such as VEGF-C and VEGF-D. (**b**) These factors promote sprouting from nearby lymphatic capillaries and vessels. (**c**) Tumor cells can become entrapped in the sprouting peripheral tumor lymphatics and stimulate dilation of the vessels leading to increased lymph flow. (**d,e**) Tumor-secreted VEGF-C and VEGF-D stimulate lymphangiogenesis and sinusoidal hyperplasia in the draining lymph nodes. (**f**) From lymph nodes, tumor cells can disseminate by traveling through the efferent lymphatic vessels and eventually drain via the right lymphatic duct and the thoracic duct into the right and left subclavian veins.

targeting VEGF-A, to the therapeutic pool of medications, the development of new cancer drugs targeting angiogenic and associated events, such as lymphangiogenesis, has been of intense interest.

8. STRATEGIES FOR INHIBITION OF LYMPHANGIOGENESIS AND TUMOR METASTASIS

With the recent identification of a number of factors associated with the development of the lymphatic vasculature and lymphatic metastasis, new strategies are being developed to inhibit cancer progression and dissemination. These include targeting of the key lymphangiogenic factors VEGF-C and VEGF-D along with their receptor VEGFR-3. Alternate opportunities for targeting consist of the other growth factors and their receptors discussed in this chapter, as well as downstream effectors. Additional therapeutic interventions target cell surface molecules such as the chemokine receptors, integrins, cell adhesion molecules, and metalloproteases, while therapeutics have been developed for intracellular targets such as COX-2, NOS, and HSP90. The main factors promoting the lymphatic dissemination of tumor cells and the agents targeting these factors are summarized in Table 1.

Table 1
Some Lymphangiogenic Molecules and Associated Therapeutics

Molecule	Targeting agent
VEGFR-3	Antibody
	Soluble receptor
	Tyrosine kinase inhibitor
VEGF-C	Antibody
VEGF-D	Antibody
VEGFR-2	Antibody
	Soluble receptor
	Tyrosine kinase inhibitor
VEGF-A	Antibody
Tie2	Soluble receptor
	Tyrosine kinase inhibitor
Angiopoietin-2	Antibody
	Peptibody
HGF/c-Met	Antibody
	Tyrosine kinase inhibitor
FGF-2	Tyrosine kinase inhibitor for receptor
IGF-1	Tyrosine kinase inhibitor for receptor

9. ANTIBODIES AGAINST VEGF RECEPTORS AND THEIR LIGANDS

Inhibition of the VEGF/VEGFR-signaling pathways can be achieved through a variety of means. While antibodies targeting VEGFR-3 interfere with the ligand-binding domain of the receptor, other antibodies under development target VEGF-C or VEGF-D. Similar to the action of bevacizumab and its target VEGF-A, these antibodies would inhibit lymphangiogenesis by attenuating VEGFR-3 signaling through interference with ligand binding *(78)*. An antibody for VEGF-D has been shown to prevent the metastatic spread of VEGF-D-expressing tumor cells to lymph nodes *(75)*. Interestingly, bevacizumab, a recombinant, humanized monoclonal antibody primarily developed for the inhibition of angiogenesis, has been shown to inhibit lymphangiogenesis as well *(79)*. The extent of this inhibition, however, has not been fully assessed in patients, and tumors expressing the mature forms of VEGF-C and VEGF-D may likely be able to evade bevacizumab treatment. Inhibition of VEGFR-3 with a monoclonal antibody has not only proven efficacious in preventing lymphangiogenesis and lymphatic metastasis *(80,81)*, but is also effective against primary tumor growth and tumor-associated angiogenesis *(82)*. Clinical trials with the VEGFR-2 antibody therapeutic IMC-1121 have been initiated with results soon to follow.

10. SOLUBLE RECEPTORS

Another means of interfering with VEGFR signaling is through the use of soluble receptors also referred to as VEGF traps. These agents are typically composed of the extracellular ligand-binding domain of the VEGF receptor fused to the immunoglobulin constant region creating high-affinity decoy receptors. Investigations with a VEGFR-3 soluble receptor capable of

sequestering VEGF-C and VEGF-D have shown that this agent inhibits not only lymphangiogenesis and lymph node metastasis but also angiogenesis in at least some tumors *(70,83,84)*. Similarly, a VEGF trap with VEGFR extracellular domain can prevent both angiogenesis and the outgrowth of LYVE-1-positive lymphatic vessels *(85)*. The outcome of clinical trials with the VEGF trap will likely soon be reported.

11. TYROSINE KINASE INHIBITORS

While biological therapeutics, such as antibodies and ligand traps, are advantageous for their specificity, the lymphangiogenic process is complex involving a number of factors. Kinase inhibitors such as those targeting the VEGFR family offer a broader approach for therapeutic intervention. These are generally pan-VEGFR inhibitors that compete for the ATP-binding site of the kinase domain and affect signaling of all three VEGFR family members as well as tyrosine kinases within the same structural class, and will prevent both angiogenesis and lymphangiogenesis. A number of these inhibitors have been developed by the pharmaceutical industry with at least two recently approved for the treatment of cancer *(86–94)*.

12. OTHER THERAPEUTIC AGENTS TARGETING VEGF-C/VEGFR-3 SIGNALING

Another antagonist of the VEGF-C/VEGFR-3 pathway is endostatin, the proteolytic fragment of collagen XVIII and endogenous inhibitor of angiogenesis *(95)*. Recently, studies showed that endostatin inhibits both lymphangiogenesis and lymphatic metastasis. While one study concluded that endostatin production by tumor cells resulted in less expression of VEGF-C by the same cells *(96)*, another study found that overexpression of endostatin in keratinocytes resulted in less aggressive carcinogen-induced squamous cell carcinomas and less accumulation of VEGF-C-expressing tumor-infiltrating mast cells *(97)*.

13. MOLECULES BLOCKING ANGIOPOIETINS AND TIE RECEPTORS

While Tie signaling is crucial for angiogenesis and lymphangiogenesis, few therapeutics have been developed targeting this pathway. Preclinical studies, however, have shown that peptide fusion proteins and an antibody targeting Ang-2 are effective antitumor and angiogenesis agents. Earlier studies showed that a soluble Tie2 molecule could also reduce tumor vascularization and primary tumor growth *(98)*. Chemical inhibitors of Tie2 have been identified and developed, but potential antitumor effects are yet to be shown *(99,100)*. Furthermore, it is unknown how these potential therapeutics may affect lymphangiogenesis and metastasis.

14. MOLECULAR TARGETING OF CHEMOKINES AND THEIR RECEPTORS

Agents targeting chemokines and their receptors currently consist primarily of CXCR4 inhibitors, which include antibodies, small-molecule receptor antagonists, and peptides. While the CXCR4 antagonist plerixafor is used to mobilize stem cells out from the bone marrow and into the circulating blood, it may also prevent tumor cells from homing to the lymphatic system thereby abrogating tumor metastasis *(101)*.

15. TARGETING OF OTHER PATHWAYS

Therapeutics have been developed targeting other factors described here and which may affect lymphatic metastasis. While studies have shown that a soluble EphB4 molecule is capable of reducing the growth of tumors expressing the receptor, its potential as an antimetastatic remains to be seen *(102,103)*. The expression of a CD44 splice variant v6 has been reported to correlate with lymphatic metastasis of human breast and lung cancers *(104,105)*. However, initial clinical studies with an inhibitory antibody targeting this molecule were halted due to poor patient outcome *(106)*. Inhibitors of metalloproteases are now under investigation for antiangiogenic and antimetastasis activity. These include the endogenous tissue inhibitor of metalloproteinase (TIMP), chemically synthesized inhibitors, and well-studied antimicrobials such as doxycycline. In addition, the inflammatory mediator cyclooxygenase 2 (COX-2) has also been shown to induce lymphangiogenesis and lymphatic metastasis in experimental tumor models *(62)*. In line with this, COX-2 inhibitors, originally developed to alleviate inflammatory conditions such as rheumatoid arthritis, can curtail the metastatic dissemination of tumor cells, and inhibit primary tumor growth possibly by preventing the recruitment of tumor-associated macrophages *(107,108)*. Other agents already in use for other conditions or diseases and which may impact lymphatic metastasis include the immunosuppressant rapamycin. The rapamycin target mTOR (the mammalian target of rapamycin) is downstream of PI3-K and Akt, which are activated through VEGFR signaling, as well as through other tyrosine kinase receptors. Therefore, it is not surprising that rapamycin can inhibit lymphangiogenesis *(109)*.

16. FUTURE PROSPECTS IN MOLECULAR TARGETING OF LYMPHANGIOGENESIS AND TUMOR METASTASIS

Most of the agents described here are still undergoing preclinical and clinical evaluation; however, the FDA has approved at least two tyrosine kinase inhibitors affecting VEGFR-3 activity *(110)*. Furthermore, there are several therapeutics already in use for other conditions that may have an impact on cancer cell dissemination through the lymph vascular system. Future identification and characterization of additional lymphangiogenic molecules will yield yet even more possible therapeutic agents. The tackling of lymphangiogenesis will likely prove useful in curtailing the metastatic progression beyond the current curative reach. As adjuvant therapy with cytostatics or other agents, antilymphangiogenics will likely prove successful at impeding the progression of cancer.

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26 Combined Targeting of EGFR and Angiogenesis in Aerodigestive Carcinomas

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CONTENTS

ABSTRACT

The epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor (VEGF) are validated molecular targets in aerodigestive carcinomas (ADCs). Effective targeting of these important molecules has been achieved with various strategies—mainly with monoclonal antibodies (mAbs) and small-molecule tyrosine kinase inhibitors (TKIs). Of particular interest is the combined use of EGFR and VEGF/VEGFR inhibitors which is based on promising early data. Here, we review the rationale and emerging clinical data with this innovative approach. The identification and validation of biomarkers that correlate with clinical outcome are expected to allow for a more rational use of EGFR and VEGF/VEGFR inhibitors. Ongoing clinical trials are expected to define the role of combined targeting in patients with nonsmall-cell lung cancer (NSCLC) and squamous cell carcinoma of the head and neck (SCCHN).

Key Words: nonsmall-cell lung cancer; head and neck cancer; esophageal cancer; epidermal growth factor receptor; vascular endothelial growth factor

1. INTRODUCTION

Carcinomas that originate from the aerodigestive tract epithelium—including head and neck, lung, and esophageal carcinomas—which are referred to as aerodigestive carcinomas (ADCs)—are the leading causes of cancer-related mortality worldwide *(1)*. ADCs are usually diagnosed at

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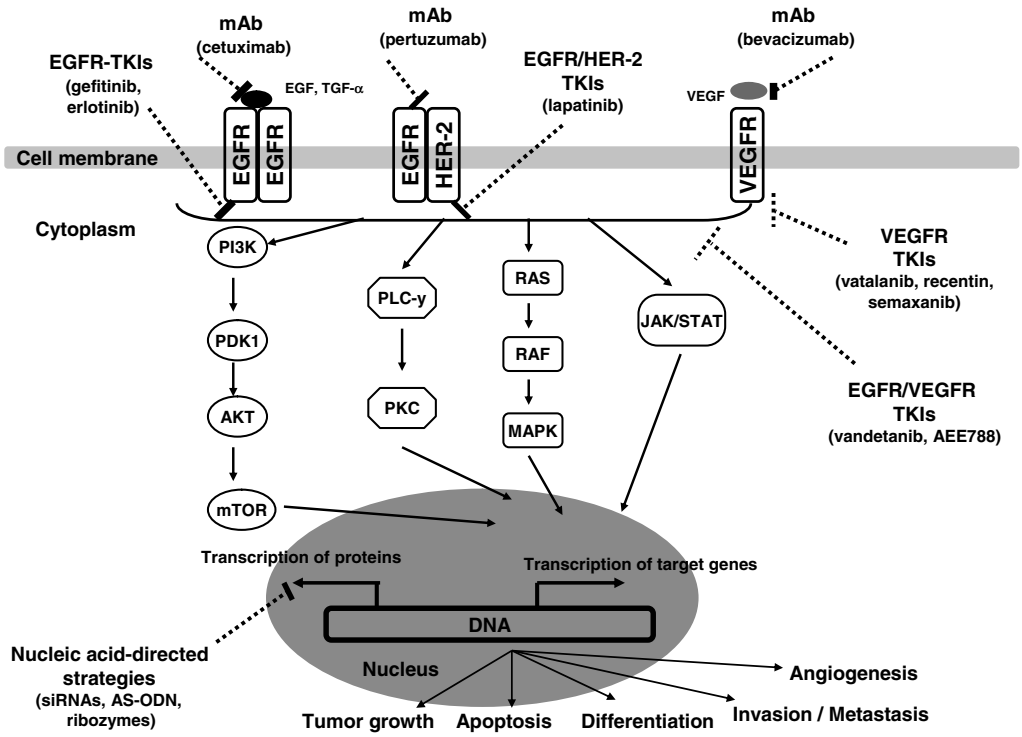

Fig. 1. EGFR and VEGFR pathways share many downstream signaling pathways and their cross-talk interaction is well established. Various strategies have been developed to inhibit these pathways, such as monoclonal antibodies (mAbs), single, dual, or multiselective tyrosine kinase inhibitors (TKIs) as well as nucleic acid-directed gene silencing molecules antisense oligodeoxynucleotides (AS-ODN) small interference RNA (siRNA).

advanced stages where traditional treatments have limited curative potential. In recent years, rationally designed therapeutic agents have been developed as a result of a deeper understanding of cancer biology. Growing evidence suggests that membrane receptor signaling pathways hold a pivotal role in carcinogenesis contributing directly or indirectly to the acquisition of malignant phenotype. Two major signaling pathways that have been successfully targeted in solid tumors are the epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) receptor pathways (Fig. 1).

2. EGFR AND VEGF/VEGFR-SIGNALING PATHWAYS

EGFR is a type I tyrosine kinase (TK) membrane receptor that regulates key cellular functions in epithelial malignancies through its signaling cascades *(2)*. Multiple studies have shown that EGFR is commonly overexpressed in nonsmall-cell lung cancer (NSCLC) *(3)*, squamous cell carcinoma of the head and neck (SCCHN) *(4)*, and esophageal cancer *(5)*. Additionally, high EGFR expression usually correlates with worse patient outcome. Moreover, a high *EGFR* gene copy number has been identified as a prognostic marker in NSCLC *(3)* and HNSCC *(6,7)*. Therapies against EGFR, mainly monoclonal antibodies (mAbs) directed against the extracellular receptor domain and small-molecule tyrosine kinase inhibitors (EGFR-TKIs), have been extensively studied in the treatment of ADC *(2)*.

Angiogenesis, the formation of new blood vessels from preexisting vasculature, is necessary for tumor growth, progression, and metastasis *(8)*. This process is regulated by proangiogenic

and antiangiogenic factors *(9)*. Tumor cells and tumor-related stroma secrete proangiogenic factors that activate endothelial cells on nearby blood vessels. The most prominent of these proangiogenic factors is VEGF *(10)*. VEGF is a member of the VEGF/platelet-derived growth factor (PDGF) family that also includes four other VEGF isoforms (VEGF-B, VEGF-C, VEGF-D, and VEGF-E) *(11)*. The VEGF family of proteins increase blood vessel permeability, endothelial cell proliferation, migration, and differentiation *(12)*. These actions are achieved with the binding of VEGF to related receptors, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1 or KDR), and VEGFR-3 (also referred to as Flt-4) which is primarily associated with lymphangiogenesis *(13,14)*. Although VEGF binds to VEGFR-2 with a lower affinity than to VEGFR-1, it has been demonstrated that VEGFR-2 is the primary mediator of VEGF-driven effects *(15,16)*. VEGF expression is enhanced by many tumor-related factors, such as oncogene expression (e.g., EGFR, RAS) and hypoxia *(17)*. Preclinical data showed both decreased angiogenesis and reduced metastatic potential by either blocking circulating VEGF or inhibiting the VEGFR pathway *(18)*. Furthermore, there is an emerging body of evidence showing improved antitumor activity when VEGF pathway inhibitors are combined with conventional cytotoxic agents *(18)*.

Solid tumors are unlikely to be entirely dependent on a single aberrant signaling pathway. Compensatory cross talk between distinct signaling pathways may provide a survival advantage for tumor cells. Preclinical data support the potential importance of cross talk between EGFR- and VEGF/VEGFR-signaling pathways, and that combined targeting results in enhanced antitumor activity *(19–24)*. Moreover, initial clinical studies have shown that combined targeting of these two molecular pathways may confer clinical benefit in patients with solid tumors *(25–27)*.

3. STRATEGIES FOR EGFR AND VEGF/VEGFR INHIBITION

Several strategies are being developed to disrupt the EGFR and VEGF signal transduction pathways. Among them, anti-EGFR and anti-VEGF mAbs as well as EGFR-TKIs and VEGFR-TKIs have undergone extensive investigation. TKIs have variable selectivity for their target TKs, while dual or multiselective TKIs have been developed, which may provide a therapeutic advantage (Fig. 1). Erlotinib, an EGFR-TKI, was evaluated in a randomized, placebo-controlled phase III trial in patients with advanced NSCLC who had previously received first- or second-line chemotherapy. Overall survival in the erlotinib arm was 6.7 versus 4.7 months in the control group ($p < 0.001$) *(28)*. Cetuximab, an anti-EGFR chimeric mAb, was studied in combination with radiotherapy in a phase III randomized study in patients with locally advanced SCCHN. The addition of cetuximab to radiotherapy conferred survival benefit (median of 49 vs. 29.3 months, $p = 0.03$) and improvement in locoregional control (median of 24.4 vs. 14.9 months, $p = 0.005$) *(29)*. Finally, a recently reported phase III trial evaluated the addition of cetuximab to platinum/5-FU for the treatment of patients with recurrent and/or metastatic SCCHN. The median overall survival was 10.1 months for patients who received cetuximab and 7.4 months for the chemotherapy-alone group ($p = 0.03$) *(30)*. EGFR inhibitors demonstrate a predictable and manageable toxicity profile. Somatic mutations in EGFR and other molecular biomarkers are currently evaluated as critical parameters for patient selection and treatment individualization.

Bevacizumab is a recombinant humanized IgG1 mAb that binds to VEGF and inhibits its activity. Bevacizumab was recently approved by the FDA when used in combination with carboplatin and paclitaxel for the first-line treatment of advanced NSCLC. This approval was based on results of a phase III randomized trial that showed a survival benefit in patients treated with carboplatin, paclitaxel, and bevacizumab compared with carboplatin and paclitaxel alone

(median survival was 12.5 vs. 10.2 months; $p = 0.0075$) *(31)*. More recently, another randomized, placebo-controlled phase III study evaluated the addition of bevacizumab (in two dosing schedules) to cisplatin and gemcitabine in the treatment of advanced NSCLC *(32)*. The median progression-free survival (PFS), which was the primary endpoint, was significantly longer with chemotherapy and bevacizumab (6.7 months on 7.5 mg/kg dose; $p = 0.002$ and 6.5 months on 15 mg/kg dose; $p = 0.03$) compared with chemotherapy alone (6.1 months). Bevacizumab is generally well tolerated but has a unique toxicity profile that may include hypertension, proteinuria, arterial thromboembolic events, impaired wound healing, bleeding complications, and gastrointestinal perforation. Due to the potential risk of fatal bleeding events, patients with squamous cell NSCLC, brain metastases, history of bleeding, or on therapeutic anticoagulation have been generally excluded from bevacizumab trials. Ongoing trials have been designed to establish the safety of bevacizumab in some of these high risk groups. In addition to mAbs, such as bevacizumab, a number of TKIs targeting angiogenesis have been developed (e.g., vatalanib, sunitinib, sorafenib) and have demonstrated clinical efficacy in various cancers *(33–36)*.

Agents targeting EGFR exert their antitumor effect directly by inhibiting tumor cell proliferation and survival, as well as indirectly, by reducing the secretion of proangiogenic growth factors, such as VEGF *(37)*. It has been reported that EGFR targeting may also modulate the function of surrounding vascular endothelial cells *(38)*. Noteworthy, the dependence of tumor vasculature on VEGF decreases as the disease progresses due to increasing redundancy of other proangiogenic pathways *(39)*. Additionally, in the presence of VEGF inhibition, some tumors may exhibit VEGF resistance via a PDGF receptor-driven escape mechanism. The PDGF receptor stimulates pericyte recruitment and activation, whereas endothelial cells are predominantly dependent on VEGF. Therefore, it has been suggested that by blocking EGFR downstream cascades it may be also possible to enhance antiangiogenic activity *(40)*. Moreover, tumors resistant to EGFR inhibitors show increased VEGF levels *(41)*. It has been suggested that VEGF overexpression may result in resistance to EGFR inhibitors due to the inability of these agents to downregulate VEGF. Theoretically, this could be overcome by combining EGFR inhibitors with agents targeting VEGF/VEGFR.

A series of preclinical studies have shown at least additive, if not synergistic, antitumor activity of the combined EGFR and antiangiogenic targeting *(42–44)*. This has been evaluated using either the combination of single EGFR and VEGFR inhibitors or the agents that are dual inhibitors (e.g., vandetanib) (Fig. 1). It should be noted that dual inhibitors may have different affinities for each receptor. Theoretically, enhanced toxicities may be encountered if drug concentration has to be significantly increased in order to achieve optimal inhibition of both targets.

The combination of antiangiogenic agents with radiotherapy has been investigated in the laboratory resulting in, at least, additive effects on tumor growth *(45–47)*. Further preclinical studies demonstrated that the triple combination of EGFR- and VEGF/VEGFR-signaling blockade and radiotherapy induced the greatest effect on tumor growth and angiogenesis *(48,49)*.

4. CLINICAL EXPERIENCE WITH DUAL TARGETING OF EGFR AND VEGF/VEGFR

4.1. Nonsmall-Cell Lung Cancer

A number of clinical trials have examined the use of bevacizumab with EGFR-TKIs—particularly erlotinib—in NSCLC (Table 1). A phase I/II trial that evaluated bevacizumab plus erlotinib in patients with advanced non-squamous cell NSCLC who had received at least one prior platinum-based chemotherapy regimen reported encouraging efficacy and safety results *(50)*. Among the 40 enrolled patients, 20% achieved partial response, and 65% stable disease. Median overall survival for the 34 patients treated at the recommended phase II dose

Table 1
Clinical Trials of Combined EGFR and VEGF/VEGFR Targeting in ADC

Phase	*Tumor*	*Treatment*	*Results*	*References*
(A) Combination of EGFR and VEGF inhibitors				
I/II	NSCLC (second line)	Bevacizumab (15 mg/kg/3 weeks) plus erlotinib (150 mg/day)	Median PFS: 6.2 months Grade 3/4 events: rash (6%), infection (9%)	*(50)*
II	NSCLC (first line)	Bevacizumab (15 mg/Kg/3 wks) plus erlotinib (150 mg/day)	Median TTP: 5.5 months Grade 3/4 events: rash (9.9%), thrombosis (1.8%), diarrhea (0.9%), hypertension (0.9%)	*(51)*
II	NSCLC (second line)	Bevacizumab (15 mg/kg/3 weeks) plus chemotherapy (docetaxel or pemetrexed) or erlotinib (150 mg/day) versus chemotherapy plus placebo	Median PFS: 4.8 versus 4.4 versus 3 months 1-year survival: 53.8% versus 57.4% versus 33.1% Toxicity of bevacizumab–erlotinib favorable compared with chemotherapy regimens	*(52)*
III	NSCLC (second line)	Bevacizumab plus erlotinib or placebo	Ongoing	NCT00130728
III	NSCLC (first line)	Chemotherapy plus bevacizumab followed by bevacizumab plus erlotinib or placebo	Ongoing	NCT00257608
II	NSCLC (first line)	Bevacizumab plus cetuximab plus paclitaxel and carboplatin	Ongoing	NCT00343291
I/II	Recurrent or metastatic SCCHN	Bevacizumab (15 mg/kg/3 weeks) plus erlotinib (150 mg/day)	Median PFS: 127 days Grade 3/4 events: diarrhea (2 pts), hemorrhage (1 pt)	*(56)*
II	Recurrent or metastatic SCCHN	Bevacizumab (15 mg/kg/3 weeks) plus cetuximab	Ongoing	NCT00407810
II	Recurrent or metastatic SCCHN	Erlotinib plus bevacizumab versus erlotinib plus sulindac	Ongoing	NCT00392665
II	Locally advanced SCCHN	Bevacizumab (10 mg/kg/2 weeks) plus erlotinib (100 mg/day) plus radiotherapy and cisplatin	Ongoing	NCT00140556

(*Continued*)

Table 1
(Continued)

Phase	*Tumor*	*Treatment*	*Results*	*References*
(B) Dual EGFR and VEGFR inhibitor (Vandetanib)				
II	NSCLC	Vandetanib (300 mg/day) versus gefitinib (250 mg/day)	Median PFS: 11 versus 8.1 weeksHigher incidence of diarrhea (58% vs. 41%), hypertension (12% vs. 1%), QT-related events (21% vs. 5%)	*(53)*
II	NSCLC	Docetaxel (75 mg/m^2) plus vandetanib (100 or 300 mg) or placebo	Median PFS: 4.4 (100 mg) and 4 (300 mg) versus 2.8 months (placebo) Common adverse events included diarrhea, rash, and asymptomatic QTc prolongation	*(54)*
II	NSCLC	Vandetanib (300 mg/day) ± carboplatin (AUC = 6 mg/ml min) and paclitaxel (200 mg/m^2)	Median PFS: 24 versus 23 weeks Higher incidence of rash (64% vs. 33%), diarrhea (53% vs. 32%), QT-related events (22% vs. 4%) and hypertension (32% vs. 4%)	*(55)*
III	NSCLC (chemotherapy and EGFR inhibitors failure)	Vandetanib (300 mg/day) versus BSC	Ongoing	NCT00404924
III	NSCLC (second line)	Erlotinib versus vandetanib	Ongoing	NCT00364351
III	NSCLC (second line)	Pemetrexed ± vandetanib	Ongoing	NCT00418886
III	NSCLC (second line)	Docetaxel ± vandetanib	Ongoing	NCT00312377
II	Locally advanced SCCHN	Docetaxel ± vandetanib	Ongoing	NCT00459043

NSCLC, nonsmall-cell lung cancer; SCCHN, squamous cell carcinoma of the head and neck; TTP, time to tumor progression; PFS, progression-free survival; AUC, area under the curve; BSC, best supportive care

level was 12.6 months, with PFS of 6.2 months. Another phase II trial of erlotinib (150 mg/day) and bevacizumab (15 mg/kg every 21 days) in 38 patients with previously untreated advanced NSCLC reported a median time to progression of 5.5 months *(51)*. A subsequent 3-arm phase II randomized study evaluated the efficacy and safety of bevacizumab in combination with chemotherapy (docetaxel or pemetrexed) or erlotinib compared with chemotherapy alone in patients

with nonsquamous cell advanced NSCLC who have received one prior chemotherapy regimen *(52)*. Median PFS was 3 months in the chemotherapy-only arm, 4.8 months in the bevacizumab plus chemotherapy arm, and 4.4 months in the bevacizumab plus erlotinib arm, while the objective response rates were 12, 12, and 18%, and the 1-year survival rates were 33, 54, and 57%, respectively. No unexpected toxicities were reported in the bevacizumab arms. Therefore, these clinical results showed that erlotinib and bevacizumab are a promising regimen and justified further study of the combination in advanced NSCLC. Ongoing phase III trials are evaluating the efficacy of the combination of bevacizumab with erlotinib as first- and second-line treatment in NSCLC (Table 1).

Vandetanib, a dual EGFR and VEGFR inhibitor, has been intensively studied in advanced NSCLC. A phase II randomized clinical trial compared single-agent vandetanib versus gefitinib, allowing crossover to the other agent at disease progression, in patients with previously treated NSCLC *(53)*. Patients receiving vandetanib had a significant prolongation of PFS compared with patients receiving gefitinib (2.6 vs. 1.9 months; $p = 0.025$). However, overall survival was not significantly different between patients randomized to either vandetanib or gefitinib (6.1 and 7.4 months, respectively; $p = 0.34$). In another, 3-arm randomized phase II trial, vandetanib, given at a dose of either 100 or 300 mg/day, plus docetaxel was compared with placebo plus docetaxel as second-line treatment in 127 patients with advanced NSCLC after failure of first-line platinum-based chemotherapy *(54)*. The median PFS was 4.4 months with 100 mg vandetanib ($p = 0.074$, vs. placebo), 4 months with 300 mg vandetanib ($p = 0.461$, vs. placebo), and 2.8 months with placebo, whereas median overall survival was 13.4, 13.1, and 7.9 months, respectively, without statistically significant differences between the vandetanib and the placebo arms. The combination regimen using the lower vandetanib dose of 100 mg daily produced a prolongation of PFS that met prespecified study criteria for further evaluation in the phase III setting. Common adverse events attributed to vandetanib in the above trials were diarrhea, hypertension, skin rash, and asymptomatic QTc interval prolongation. Additionally, another 3-arm randomized phase II trial evaluated the efficacy of vandetanib with carboplatin and paclitaxel, as well as vandetanib alone versus carboplatin and paclitaxel alone in the first-line treatment of advanced NSCLC setting versus chemotherapy alone and vandetanib alone *(55)*. The vandetanib monotherapy arm was terminated early because of inferior PFS at interim analysis (HR > 1.33 vs. carboplatin/paclitaxel). The median PFS with vandetanib/carboplatin/paclitaxel and carboplatin/paclitaxel alone was 5.6 and 5.4 months, respectively. The primary objective of the study was met, with the combination schedule prolonging PFS compared with chemotherapy (HR = 0.76, 95% CI 0.50–1.15; $p = 0.098$). Overall survival, a secondary endpoint, was not significantly different between the two treatment arms (HR = 1.07, 95% CI 0.63–1.81; $p = 0.595$). The objective response rates were 32, 25, and 7% for vandetanib/carboplatin/paclitaxel, carboplatin/paclitaxel, and vandetanib, respectively. Currently, a number of phase III trials have been initiated to test the efficacy of vandetanib either as monotherapy or in combination with standard chemotherapeutic agents in advanced NSCLC (Table 1).

4.2. Squamous Cell Carcinoma of the Head and Neck

Encouraging results have been observed with the combination of bevacizumab and erlotinib in a phase I/II clinical trial in recurrent or metastatic SCCHN *(56)*. In the phase I part of the trial bevacizumab was safely escalated to 15 mg/kg every 3 weeks in combination with erlotinib 150 mg daily. A total of 48 patients were treated in the subsequent phase II study. The objective response rate was 15%, the median PFS 3.8 months, and the median overall survival 6.8 months. Toxicities were expected based on the single-agent toxicity profile. In the phase II part of the study, three bleeding events were noted, one fatal *(56)*. The ratio of phosphorylated VEGFR-2/

VEGFR-2 was proposed as a predictive factor of the combined EGFR and VEGF targeting *(57)*. An ongoing phase II study is evaluating the safety and efficacy of the combination of cetuximab with bevacizumab in recurrent or metastatic SCCHN (Table 1). The combined targeting of EGFR and VEGFR pathways with irradiation has shown additive effects in SCCHN xenografts *(49)*. Clinical studies are testing the incorporation of the combined EGFR–VEGF/VEGFR targeting in standard chemoradiotherapy regimens of locally advanced SCCHN (Table 1).

5. CONCLUSIONS AND FUTURE PERSPECTIVES

As solid tumors are characterized by multiple, and often redundant and cross talking, deregulated signaling pathways, it is unlikely that targeting a single pathway will suffice in achieving optimal antitumor activity. Combined targeting of two or more critical pathways may be a more efficacious approach, which could potentially counteract escape mechanisms for cancer cell growth and survival. Specifically, combined inhibition of EGFR and VEGF/VEGFR is an attractive approach for cancer therapy, which is favored by a strong preclinical rationale and emerging clinical data in NSCLC and SCCHN. The study of similar concepts in esophageal carcinomas is warranted. The identification of biomarkers predictive of clinical outcome may provide tools for better patient selection for treatment with innovative molecular targeted agents. Recently, data have suggested a number of potential biomarkers of antiangiogenic activity, including plasma VEGF, serum levels of VEGFR-2, circulating endothelial cells (CECs), and endothelial progenitor cells (EPCs). Novel imaging techniques, such as dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), PET, and dynamic CT scans, may contribute to the understanding of mechanism of action of antiangiogenesis agents *(12)*. The evaluation of biomarkers in clinical trials employing combined EGFR–VEGF/VEGFR targeting is currently ongoing *(55,58,59)*. Finally, the acute and late toxicity profile of these combinations should be carefully monitored. In conclusion, combined targeting of EGFR and angiogenesis represents a promising innovative approach. The merits of incorporation of these strategies into the standard management of patients with ADC and other malignancies will be definitively addressed by ongoing and planned clinical investigations.

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27 Targeting Signaling Pathways in Cancer Therapy

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CONTENTS

ABSTRACT

The clinical results of treatment with traditional chemotherapy for most solid tumor malignancies have remained disappointing over the last decades. Toxicities are often prohibitive. This demands the development of innovational, systemic treatment approaches. Small-molecule tyrosine kinase inhibitors (sm-TKIs) have shown promising results in several malignancies. We are reviewing the current progress of cancer therapy with sm-TKIs in solid tumor malignancies with a focus on breast cancer, hepatocellular cancer, colon cancer, and nonsmall-cell lung cancer and place this progress into the context of improvements in recent, targeted antineoplastic therapies.

Key Words: tyrosine kinase inhibitors; small molecules; solid tumor malignancies; targeted therapy; EGFR inhibition; response predictors; breast cancer; hepatocellular cancer; lung cancer; colon cancer

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1. INTRODUCTION

Targeted therapy, utilizing pharmacokinetic and pharmaco-dynamic differences between normal and malignant cells *(1)*, has been investigated for several decades. The National Cancer Institute subclassifies these approaches into the following categories *(2)*:

- Small-molecule drugs
- Monoclonal antibodies (MOABs)
- Gene therapy
- Apoptosis-inducing drugs
- Angiogenesis inhibitors
- Cancer vaccines

This review will focus on the clinical application of the first subcategory, the small molecules, with an emphasis on small-molecule tyrosine kinase inhibitors (sm-TKIs). Major clinical advances have been made utilizing these drugs, particularly in nonsmall-cell lung cancer (NSCLC), hepatocellular cancer (HCC), and breast cancer. We will provide a clinical review of these interesting observations. For a more comprehensive review of the molecular targets of the targeted approach, refer to our previous publications *(3,4)*.

2. COMMON BIOLOGICAL TARGETS

There are several common biological structures that are important in targeted antineoplastic therapies. We will review these structures and their relevance in malignant transformation, tumor growth, and metastasis in brief. For a more comprehensive review, refer to previous publications on this subject *(3,4)*.

The HER family of molecules are expressed in all cells of epithelial origin and play a role in normal cellular activities *(3,5)*. Members of the *HER* family include *HER1*(epidermal growth factor receptor—EGFR), *HER2* (*erbB2, HER2/neu*), *HER3*, and *HER4*. Overexpression has been documented in numerous epithelial malignancies and its activation appears to promote the development and progression of malignancy. All members of the *HER* family share a common extracellular ligand-binding domain, a single membrane-spanning region, and a cytoplasmic TK domain *(6,7)*. A ligand for HER2 has not been identified, while HER3 lacks TK activity. Ligand binding to HER1, HER3, or HER4 induces these inactive monomers to undergo an array of homo- or heterodimerization with other members of the HER family. HER2 is the preferred heterodimeric partner for all HER receptors, resulting in a complex that is endocytosed at one half to one third the rate of other HER dimmers *(8,9)*. HER dimerization leads to receptor autophosphorylation and subsequent activation of the TK domain. The signaling characteristics of the HER family are thought to be strongly interdependent.

EGFR overexpression has been described in many cancer types. Therefore, EGFR is a rational target for antineoplastic treatment. Intrinsic ligands include transforming growth factor-α (TGF-α) and epidermal growth factor (EGF). Binding to the receptor leads to its homo- or hetero (with other members of the erb family)-dimerization and subsequent intracellular transphosphorylation of the tyrosine domain *(10)*. Downstream target proteins include mitogen-activated protein kinase (MAPK), phosphotidylinositol-3 kinase (PI3K), and the signal transducer and activator of transcription (STAT) family of proteins. These proteins are tightly linked to cell proliferation (MAPK), cell-cycle progression and survival (PI3K), and cell division, survival, invasion, and adhesion (STAT) *(11)*. Of the multiple EGFR-family-targeted therapeutic strategies, sm-TKIs and MOABs are the best studied.

While activating mutations of the EGFR receptor have been described in NSCLC *(12,13)*, such activation patterns are not necessarily observed in other solid tumors *(14)*. However, elevated EGFR levels and its ligands may be associated with tumor aggressiveness and poor prognosis in pancreatic cancer *(15)*.

Another family of TKs that has been found to bear significant relevance in cancer pathogenesis, growth, and metastasis is the vascular endothelial growth factor family (VEGF). VEGF belongs to a major family of receptors, including VEGFR-1 and -2 as well as placental growth factor (PlGF) *(16)*.

VEGF and its isoforms are the ligands for VEGF receptors (VEGFR)-1 and -2 (Fig. 1). Although VEGF binds to two receptor TKs, VEGFR-1 and/or VEGFR-2, the angiogenic effects are primarily exerted through binding to VEGFR-2. *(17)* The binding of VEGF to these receptors on endothelial cells results in receptor dimerization and activation, which stimulates signaling cascades involving phospholipase C, protein kinase C, the Src TKs, MAP kinase, PI3K, Ras GTPase-activating protein, and the Raf-Mek-Erk pathway (reviewed in *(18,19)*) (Fig. 1). Additionally, VEGF binds to the non-TK neuropilin receptors (NRP-1 and NRP-2). NRP-1 can act as a coreceptor for VEGF and as such potentiates VEGFR-2-dependent endothelial cell mitogenesis (Fig. 1).

This inhibition of cell-cycle processes, essential for the survival of cancer cells, makes VEGF as well as HER inhibition a treatment strategy attractive for both solid tumor and hematological malignancies. Secondary to the structural similarities of the different TKs, most TKIs are not specific, but rather demonstrate a promiscuous inhibition of different target TKs. Table 1 lists multiple TKIs. Note that only some of them were of proven clinical benefit in phase III trials. The majority of those agents are still under preclinical or clinical investigation.

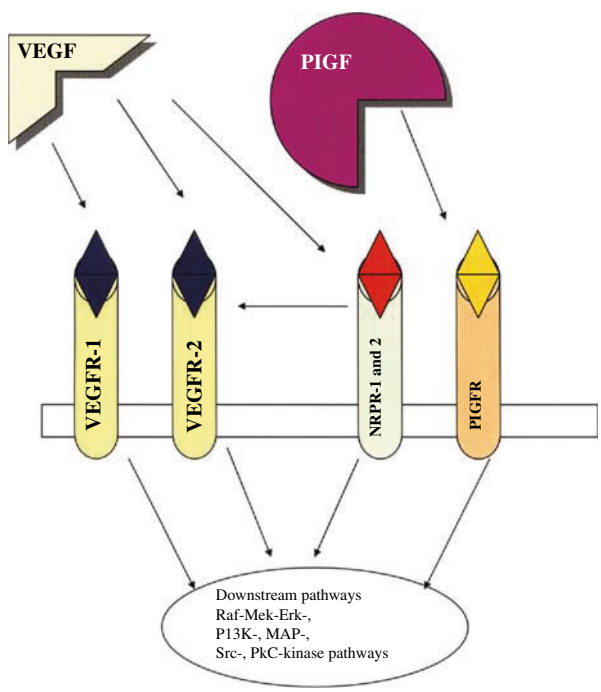

Fig. 1 The figure above describes the interaction of placental growth factor (PlGF) and vascular endothelial growth factor(VEGF) with their receptors

Table 1
Small-Molecule-Targeted Agents Used

I. Membrane-based receptor inhibition

A. Small-molecule type EGF (erb-1) receptor TKIs
1. Gefitinib
2. Erlotinib
3. EKB-569
4. PKI-166
5. Lapatinib

B. Small-molecule type VEGF TKI
1. Vatalanib (PTK787)
2. Semaxanib (SU5416)
3. ZD4190
4. AZD2171
5. CEP4214
6. CEP7055
7. AG0137336

C. PDGFR inhibition and multitargeting drugs
1. Imatinib
2. Sutent (SU11248)
3. Sorafenib (BAY43-9006)
4. AG013736
5. Vatalanib (PTK787)
6. Leflunomide
7. THI-31

D. Combined VEGFR and EGFR inhibition
1. Vandetanib (ZD6474)
2. AEE 788

II. Cytoplasmatic TK inhibition
1. PP1
2. PP2
3. AZM475271
4. siRNA
5. AP23846
6. SKI-606
7. AZD05230

3. MONOCLONAL ANTIBODIES AND SMALL-MOLECULE TKLs

Very limited evidence comparing small-molecule TKIs with MOAB therapy is available. The conclusion derived from these comparative studies is largely based on circumstantial evidence. However, several lessons have been learned from the trials involving targeted antineoplastic therapies.

Firstly, four large phase III trials in patients with NSCLC compared chemotherapy alone to the same chemotherapy with TKIs. These trials did not reveal any benefit to adding the TKI to

conventional chemotherapy *(20–23)*. The INTACT-1 trial enrolled and randomized 1,093 patients. Up to six cycles of chemotherapy (cisplatin *[80* mg/m^2] on day 1 and gemcitabine [1,250 mg/m^2] on days 1 and 8 of the 3-week cycle) plus either gefitinib (500 mg/day) or gefitinib (250 mg/day) were given to chemonaive patients. The INTACT-2 trial, which included 1,037 patients, utilized a chemotherapy more frequently utilized in the USA. Patients received paclitaxel 225 mg/m^2 and carboplatin area under concentration/time curve of 6 mg/min/ml (day 1 every 3 weeks) plus gefitinib (500 mg/day), gefitinib (250 mg/day), or placebo. After a maximum of six cycles, daily gefitinib or placebo continued until disease progression. None of the two trials detected significant differences in time to progression (TTP), overall survival (OS), or response rates *(20,21)*.

Similar trials were also performed utilizing erlotinib as the TKI. The TRIBUTE trial reported 1,059 assessable patients with untreated stage IIIB/IV NSCLC with good performance status, who were randomly assigned to erlotinib (150 mg/day) or placebo combined with up to six cycles of carboplatin and paclitaxel, followed by maintenance monotherapy with erlotinib *(22)*. The Tarceva Lung Cancer Investigation Trial, lead out of Germany, utilized up to six 21-day cycles of chemotherapy (gemcitabine [1,250 mg/m^2] on days 1 and 8 and cisplatin 80 [mg/m^2] on day 1 with or without erlotinib [150 mg/day] *[23]*). Again, both trials did not show a difference in survival, TTP, or response rate *(22,23)*.

In contrast, the combination of the monoclonal VEGF antibody bevacizumab with paclitaxel (200 mg/m^2) plus carboplatin Area Under Curve (AUC 6) showed a statistically significant survival benefit in patients with a good PS (0 or 1) and either stage IIIB (with a malignant pleural effusion) or stage IV disease *(24)*.

Even more interesting are the early press publications of the FLEX trial (First-Line Treatment for Patients with Epidermal growth factor inhibitor [EGFR]-EXpressing Advanced NSCLC) *(7)*. This large, randomized multinational study on patients with stage IIIB or stage IV NSCLC who had not previously received chemotherapy investigated the combination of cisplatin/vinorelbine plus/minus the addition to cetuximab. The biologic target is similar to the INTACT and TRIBUTE trials *(20–23)*. In contrary to the TKI though, the actual study endpoint—OS, was met according to the early press release *(25)*.

Secondly, positive results have been observed for the combination of MOABs with chemotherapy in colorectal cancer (CRC) *(26)*. In the BOND-1 trial, a randomized phase II study in patients with irinotecan-refractory metastatic CRC, all patients were naive to cetuximab. The rate of response in the combination therapy group was significantly higher than that in the monotherapy group (22.9 vs. 10.8%; $p = 0.007$). The median TTP was significantly greater in the combination therapy group (4.1 vs. 1.5 months, $p < 0.001$ by log-rank test). The median survival showed a statistically nonsignificant trend (8.6 vs. 6.9 months) favoring the combination cetuximab/CPT-11 vs. CPT-11 alone. Again, the BOND-1 experience was evidence for a strategy favoring MOAB with chemotherapy as opposed to chemotherapy alone *(27)*.

No randomized trial evidence is available comparing the combination of TKIs/chemotherapy with chemotherapy alone in this disease.

Thirdly, the only large-scale randomized controlled trial evidence substantiating a role of a TKI with standard chemotherapy exists in pancreatic cancer. In the Pa.3 trial the combination of gemcitabine and erlotinib has shown to provide a statistically significant survival benefit. In this phase III trial *(28)* from the National Cancer Institute of Canada gemcitabine (1,000 mg/m^2 weekly) with and without erlotinib (100 mg daily) was compared in 569 patients with locally advanced or metastatic pancreatic cancer. Combined therapy was associated with few objective responses, but OS was significantly better compared to gemcitabine alone (hazard ratio 0.81, $p = 0.038$) with about 2 weeks absolute survival benefit *(28)*. Although a 2-week survival benefit is of questionable clinical significance, particularly considering that the costs for this additional

treatment per gained year for life are beyond what is usually accepted in the USA, erlotinib/gemcitabine is now Food and Drug Administration (FDA) approved for metastatic/nonresectable pancreatic cancer *(29)*. In contrast, cetuximab was not shown to be of benefit in pancreatic cancer. Although a phase II trial utilizing cetuximab at an initial dose of 400 mg/m *(2)*, followed by 250 mg/m *(2)* weekly for 7 weeks with gemcitabine at 1,000 mg/m *(2)* for 7 weeks, followed by 1 week of rest showed promising results *(30)*, an interim report from a phase III trial *(31)*, comparing a similar regimen with gemcitabine single-agent therapy did not show statistically significant results. In a preliminary report the objective response rates (12 vs. 14%), progression-free survival (PFS) (3.5 vs. 3 months), and OS (median 6.4 vs. 5.9 months) were about the same in the gemcitabine single-agent therapy and the combination therapy arm *(31)*. However, the point estimate of the difference in OS was 0.5 months for combined gemcitabine/cetuximab and might be larger than the one from the Canadian Pa.3 trial if statistical significance was reached.

In conclusion, drug companies have little interest to compare the MOAB and TKI products targeting the same biological target, for obvious reasons. Hence, no industry-sponsored phase II trials comparing those two strategies are expected in the near future. The circumstantial evidence that is available, as further elaborated above, suggests that in certain malignancies MOABs seem to confer at least an additive benefit when combined with conventional chemotherapy. This is probably most prominent in NSCLC. In contrast, the small-molecule TKIs are in most malignancies best used as single agents. Future research into how to appropriately integrate these biological agents into oncological care (i.e., concomitant administration vs. sequencing and dose optimization) will help to determine optimal treatment in many malignancies.

4. SMALL MOLECULES IN HEPATOCELLULAR CANCER

HCC represents worldwide the third most common cause for cancer mortality and is globally the most frequent lethal gastrointestinal (GI) tumor *(32)*. It remains a major therapeutic challenge secondary to the poor response to conventional chemotherapeutic strategies what may be in part due to the high rate of expression of drug resistance genes, including p-glycoprotein, glutathione-*S*-transferase, heat shock proteins, and mutations in p53 *(33–36)*. Hence, HCC has been for long the subject of intense exploration of alternative biologic agents *(37)*. Preclinical studies revealed abnormalities in several key pathways, including VEGF *(38)*, PDGFR (platelet-derived growth factor receptor) *(39)*, EGFR *(40)*, PI3K *(41)*, STAT *(42)*, mTOR (mammalian target of rapamycin) *(43)*, HIF-1α (hypoxia-inducible factor-1α) *(41)*, and several others *(40)*.

4.1. Sorafenib

The complex carcinogenesis in HCC triggered interest in the exploration of promiscuous, multitargeted TKIs. Sorafenib is the most widely investigated targeted treatment strategy. In several preclinical studies it has been shown to inhibit the TK components of the VEGF receptors 2 and 3, PDGFRβ as well as more downstream pathways like the Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase (Raf/MEK/ERK)-signaling pathway *(44)*. In several preclinical evaluations a dose-dependent tumor response was identified *(45)*. Those encouraging preclinical results lead to a phase II trial, in which 137 patients with advanced, inoperable HCC received single-agent sorafenib at a dose of 400 mg twice daily. Partial response (PR), minor responses, and stable disease (SD) were observed at a frequency of 2.2, 5.8, and 34% respectively *(46)*. In that study, the median TTP was 4.2 months, the median OS 9.2 months. The most commonly observed grade 3–4 toxicities were fatigue (9.5%), diarrhea (8%), and palmoplantar erythrodysaesthesia (5.1%) *(46)*.

This early demonstration of clinical antitumor activity of sorafenib in HCC and the strong basic science evidence suggesting a role of the sorafenib-inhibited pathways in the carcinogenesis of HCC lead to a large international randomized phase III trial, the SHARP trial (Sorafenib HCC Assessment Randomized Protocol) *(47)*. Here, 602 patients with child A cirrhosis and Eastern Cooperative Oncology Group (ECOG) performance status of 0–2 with newly diagnosed, previously untreated HCC were randomized to sorafenib 400 mg orally twice daily or placebo. Seven patients achieved a PR (2.3%) and 211 SD (71%) and 54 (18%) showed primary disease progression. The median OS was 46 weeks in sorafenib-treated patients compared with 34 weeks in patients who received placebo. This indicated a 44% increase in OS. The median TTP was 24 weeks in sorafenib-treated patients compared with 12 weeks in patients in the placebo group, indicating a 73% prolongation in the TTP. Both outcome measures (OS and TTP) were statistically significantly improved with sorafenib therapy. Those favorable results revealed sorafenib as first effective medical therapy for HCC and as first-line therapeutic gold standard in advanced, inoperable HCC. The SHARP trial has so far just been presented in abstract form, in the plenary session of the American Society of Clinical Oncology (ASCO) 2007 meeting *(47)*. Several points of criticism, like questionable compliance with the study medication, lower than expected adverse events and the unpublished amount of minor responses in the study and control arm, will hopefully be clarified when the trial will be published in manuscript form.

4.2. Sunitinib

Suntinib is another TKI that has been, like sorafenib, widely used in renal cell malignancies. It has been shown to inhibit VEGFR types 1 and 2 (fms-related TK 1 [FLT1] and the FLK1 kinase insert domain receptor [FLK1/KDR]); PDGFR-α, and PDGFR-β, the stem cell factor receptor c-KIT, and the FLT3 and RET kinases *(48)*. Two phase II trials, utilizing sunitinib in advanced HCC have been reported in abstract form. The first trial used a dosing schedule of 37.5 mg once daily on a 4 weeks on/2 weeks off schedule *(49)*. Of the 26 enrolled patients one had a PR response of 10 months duration and ten patients (38.5%) had SD that lasted$\geq$12 weeks. The median PFS in this cohort was 4.1 months. The investigators in the second trial used a dosing schedule of 50 mg once daily *(50)*, 4 weeks on/2 weeks off was used. Thirty-seven patients were enrolled. One PR was noticed and fourteen patients had SD. None of the two investigations reported mortality results. The toxicity described was tolerable. Further research will be required to define the role of sunitinib in the management of HCC.

4.3. EGFR Tyrosine Kinase Inhibitors

Gefitinib *(51)*, erlotinib *(52,53)*, and the dual EGFR/Her2-neu TKI lapatinib *(54)* have been investigated in HCC with modest results. Gefitinib was evaluated in the cooperative group setting. The ECOG trial which used an oral dose of 250 mg gefitinib *(51)* twice daily did not enter its planned second phase when the PFS was reported to be only 2.8 months and the OS only 6.5 months. Erlotinib was evaluated in two single-armed trials *(52,53)* with limited response rates and PFS and OS ranging from 3.1 to 3.2 and 6.3 and 13 months, respectively. A California Consortium (CCC-P) trial *(54)* using lapatinib at a daily dose of 1,500 mg demonstrated two PRs in the first 17 patients enrolled. However, the PFS was only 2.3 months in all 30 patients enrolled in the trial.

4.4. Conclusion

The multitarget TKI sorafenib has shown convincing results in the treatment of advanced, inoperable HCC and can be considered first-line standard in this setting. More specific

TKIs have just demonstrated modest responses. Future results of further trials are awaited and will hopefully help to define further successful treatment options in hepatocellular malignancies.

5. SMALL-MOLECULE TKIs IN NONSMALL-CELL LUNG CANCER

The survival for NSCL cancer remains poor *(55)*. This clearly calls for new treatment strategies. Cellular growth in NSCLC has been shown to depend on the disarrangement of membranous receptor-triggered cellular pathways *(56)*. The EGFR pathway gained particular attention in recent years.

The two small-molecule inhibitors gefitinib and erlotinib have been widely preclinically and clinically investigated in the management of NSCLC. Both obtained FDA approval for second-line therapy of NSCLC.

Different response predictors have been studied with evidence mainly from phase II trials. Several clinical predictors such as Asian ethnicity, never, or oligosmoker status and female gender in conjunction with the presence of an adenocarcinoma histology (particularly bronchoalveolar) have proven to be valuable clinical response predictors *(57,58)*.

Several molecular response predictors for EGFR-directed TKI therapy have been identified over the last years. The activating EGFR mutations are the best studied. Exon 19 deletions and the L858R point mutation represent 85% of all identified EGFR mutations. They have been linked to better response and prolonged survival *(59–61)*. Interestingly, patients treated on gefitinib or erlotinib trials with exon 19 deletions did have a significantly longer survival (34–38 months) than patients with the L858R mutation (8–17 months) *(60,61)*.

EGFR overexpression, gene amplification, and other molecular markers have been shown in preclinical models and phase II trials to predict the response to small-molecule TKIs *(62–66)*. However, the significant predictive value of those molecular markers has been challenged in multivariate analyses of larger trials *(67)* and is hence under further prospective analysis in a prospective trial led out of Canada. The novel molecular techniques to identify patients more likely to respond to EGFR–TKIs is the matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) technique. Recent algorithms utilizing this technique showed promising results *(63)*.

In addition to molecular markers predicting a positive response, several molecular changes have been correlated to an absence of a response to EGFR-directed TKI therapy.

The most prominently studied are K-ras *(69)* and T790M *(70)* mutations as well as exon 20 insertions *(71)*.

Erlotinib was shown in a randomized, placebo-controlled phase III trial *(72)* to improve survival in previously treated patients with stage IIIB or VI NSCLC. Patients were eligible if they had an ECOG performance status of 0–3 and had received one or two prior chemotherapy regimens. The patients were stratified according to center, performance status, response to prior chemotherapy, number of prior regimens, and prior platinum-based therapy and were randomly assigned in a 2:1 ratio to receive oral erlotinib, at a dose of 150 mg daily, or placebo. The median age of the 731 patients who underwent randomization was 61.4 years; 49% had received two prior chemotherapy regimens and 93% had received platinum-based chemotherapy. The response rate was 8.9% in the erlotinib group and less than 1% in the placebo group ($p < 0.001$). Five percent of the patients in the erlotinib arm had to discontinue treatment secondary to limiting toxicities. The median duration of the response was 7.9 months in the erlotinib and 3.7 months in the placebo group. PFS was 2.2 months and 1.8 months, respectively, with an adjusted p value of <0.001. OS was 6.7 months in the erlotinib arm and 4.7 months in the placebo arm, what reached statistical significance *(72)*.

Several first-line metastatic lung cancer studies such as the TRIBUTE and the TALENT trials *(73,74)*, which utilized a combination of erlotinib with chemotherapy, have been performed. None of those trials showed a clear, statistically significant survival benefit. Of note is, that in a precontemplated subgroup analysis of the TRIBUTE trial oligo- and nonsmokers experienced a statistically significant survival benefit when erlotinib was added to combination chemotherapy. The absence of a detectable survival benefit for the combination of EGFR TKIs with chemotherapeutic agents may be explained by the tyrosine kinase inhibition-induced suppression of intracellular signal transmission pathways such as MAP kinase, STAT, and PI3K and the corresponding depression of cell cycle and proliferative activity. By removing cells from the cell cycle, TKIs could potentially exert a functional antagonism in conjunction with chemotherapy.

The second FDA-approved small-molecule EGFR TKI, gefitinib, received approval on the basis of clinical benefit detected in phase I/II trials including pretreated, symptomatic patients *(75)*. A subsequent large-scale phase III trial failed to detect a statistically significant OS benefit. A planned subgroup analysis including never smokers and patients of Asian ethnicity demonstrated a statistically significant survival benefit of 8.9 versus 6.1 and 9.5 versus 5.5 months for the erlotinib arm. The subsequent INTACT-1/2 phase III trials failed to reveal a survival benefit for the combination of chemotherapy with gefitinib *(76,77)*.

The combination of EGFR inhibition in combination has received recent attention. Initial phase I/II studies of erlotinib and bevacizumab achieved response rates of 20% with an SD rate of 65% *(78)*. ZD6474 is a small-molecule TKI with antagonistic activity on the EGFR TK as well as VEGFR TK *(79)*. A differential activity is hypothesized with primarily anti-VEGFR activity at lower doses and more EGFR antagonism at higher doses *(80)*. Initial phase II studies comparing ZD6474 versus gefitinib showed encouraging results reaching statistical significance for prolongation of TTP favoring ZD6474 in comparison with gefitinib *(81)*.

A study by Heymach *(82)* is now available in manuscript form. Two dose levels of ZD6474 (vandetanib 100 vs. 300 mg/day) in combination with docetaxel (75 mg/m^2 intravenous infusion every 21 days) versus docetaxel alone *(82)* were compared in a phase II randomized trial. A modest trend toward a prolongation in PFS was observed in the vandetanib 100 mg/day plus docetaxel arm. The PR rate was 18, 26, and 12% in the vandetanib 300 mg/day plus docetaxel, vandetanib 100 mg/day plus docetaxel, and docetaxel alone arm, respectively. PFS and OS were 17 weeks and 7.9 months, 18.7 weeks and 13.1 months, and 12.0 weeks and 13.4 months in the vandetanib 100 mg/day plus docetaxel arm. The PR rate was 18, 26, and 12% in the vandetanib 300 mg/day plus docetaxel, vandetanib 100 mg/day plus docetaxel, and docetaxel alone arm, respectively *(82)*. In summary, vandetanib seems to have modest biological activity in NSCLC and the optimal strategy to use this drug is still under study. Currently two phase III trials *(83,84)* are ongoing, combining vandetanib with chemotherapy versus chemotherapy alone. Two of the FDA-approved gold standards for second-line treatment in NSCLC are evaluated in those trials, docetaxel with vandetanib versus docetaxel alone *(83)* and pemetrexed with vandetanib versus pemetrexed alone *(84)*. Two other phase III trials investigate vandetanib as single agent *(85,59)*. One trial compares vandetanib with best supportive care in patients who failed prior EGFR-directed TKI treatment *(85)* and the second one compares vandetanib with erlotinib in second-line NSCLC treatment *(59)*. The results of those trials are eagerly awaited.

Another small molecule with specificity for the VEGFR TK, AZD2171 *(86)*, is currently under investigation in a phase II trial comparing carboplatin/paclitaxel/AZD2171 with carboplatin/paclitaxel alone.

6. SMALL MOLECULES IN BREAST CANCER

Biologically targeted therapy with small molecules has been investigated in breast cancer *(87,88)*. As target of particular interest is the erb2 (HER2/neu) receptor *(89)*. Aberrant signaling via this network has been linked to a worse outcome in metastatic as well as localized breast cancer, rendering targeting of this receptor system as an attractive therapeutic approach *(90)*. The EGFR(erb1) receptor, another member of the erb family, is expressed in 14–91% *(91)*, whilst overexpression has been suggested in about 27–30% of breast cancer specimens *(92,93)*. Although the prognostic value of EGFR expression remains a matter of debate, abnormal EGFR signal transduction has been consistently linked to acquired antihormone resistance in preclinical models *(94,95)*. Those preclinical findings led to the investigation of targeted small-molecule therapy of breast cancer in clinical trials.

6.1. Gefitinib and Other EGFR-Specific TKIs

Gefitinib is a reversible, highly EGFR-specific TKI *(96)*. Along with inhibition of EGFR-overexpressing cell lines, growth inhibition was noticed in HER2-overexpressing cell lines. This was attributed to the inhibition of erb1/erb2 heterodimer phosphorylation *(97)*.

Overall, the treatment of metastatic, heavily pretreated breast cancer with small-molecule anti-EGFR therapy has proved relatively disappointing *(98,99)*. Preclinical evidence of efficacy of gefitinib in tamoxifen-resistant cell lines, in conjunction with the observation that EGFR expression as well as downstream-signaling pathways is upregulated in tamoxifen-resistant breast cancers *(100,101)*, gave rise to the Iressa 57 trial *(102)*. Here, a clinical benefit was observed in the tamoxifen-resistant estrogen receptor (ER)+ subpopulation. Based on those observations the Iressa 223 trial was conducted and recently reported *(103)*. This trial was designed to investigate if gefitinib confers additional benefit when applied neoadjuvantly together with the aromatase inhibitor anastrozole. Anastrozole (1 mg/day) alone was compared with anastrozole + gefitinib (250 mg/day) as neoadjuvant therapy for breast cancer. Postmenopausal women with stage I–IIIB breast cancer and ER and/or progesterone receptor (PgR)+ tumors received anastrozole for 16 weeks and were randomized to a combination with gefitinib for 16 weeks (AG), placebo for 2 weeks, then gefitinib for 14 weeks, or placebo for 16 weeks. Biopsies were taken at baseline, then at 2 and 16 weeks. Study endpoints were change in Ki67 and tumor response after the completion of the neoadjuvant treatment course. Two hundred and six patients were randomized. Neither the biological, Ki67 suppression, nor the clinical activity of anastrozole was enhanced by the addition of gefitinib. A trend against the combination, in favor of anastrozole single-agent therapy, was noticed in this trial *(103)*. In conclusion, though the concept of anti-EGFR-directed therapy in breast cancer remains appealing, clinical evidence for efficacy of gefitinib in this disease has so far been disappointing and mainly anecdotal for erlotinib *(104)*.

6.2. Lapatinib

Whilst erb1 overexpression is observed in about 27–30%, erb2 is overexpressed in about 20–25% of all newly diagnosed breast cancers *(105,106)*. Receptor dimerization is necessary for the downstream activation of erb1 and erb2. erb1 and erb2 are common heterodimerization partners *(92,107)*. Targeted therapy of the erb2 receptor with the MOAB trastuzumab has been firmly incorporated into the treatment of truly erb2-overexpressing metastatic and early stage breast cancer *(108–111)*. Lapatinib is a TKI with dual activity, targeting the erb1 as well as the erb2 receptor *(112)*. There are several theoretical advantages of this small molecule over MOAB

therapy. First, as small molecule it has a far smaller likelihood to generate an immunogenic response. Second, as dual, erb1 and erb2 TKI, it has potentially a stronger efficacy than an inhibitor acting on erb2 alone *(113)*. Third, as small molecule it has the potential to cross the blood–brain barrier *(114)*. Last, it inhibits the truncated form of erb2, the erb2p95 TK *(115)*. This truncated form escapes treatment with trastuzumab and might confer a worse prognosis than the erb2 wild type *(116)*.

It has been shown to be active in several preclinical breast cancer models and synergy with tamoxifen has been demonstrated *(93,117)*.

During initial clinical trials lapatinib was administered to highly pretreated, chemotherapy, and trastuzumab refractory patients *(116,118)*. Most patients in the first two phase II trials had erb2-overexpressing disease, whilst the EGF20008 study also included an arm of not truly erb2-overexpressing not trastuzumab-resistant disease. The clinical benefit rates, consisting of all patients with SD as well as PR and complete response, were reported as 22 and 14% in the EGF20002 *(116)* and EGF20008 trial *(118)*, respectively.

After phase I evidence established the combination of capecitabine and lapatinib as safe *(119)*, a phase III trial comparing capecitabine alone with the combination with lapatinib was conducted *(114)*.

Eligible patients had ErbB2-overexpressing advanced or metastatic breast cancer. Prior anthracycline, taxane, and trastuzumab therapy was required for inclusion. Patients were randomized to receive lapatinib 1,250 mg once daily with capecitabine 2,000 mg or 2,500 mg/m^2 every day on days 1–14. In the combination therapy arm the TTP and PFS was 37 weeks versus 20 and 18 weeks only for TTP and PFS in the capecitabine arm. Both endpoints showed a statistically significant improvement intelapatinib/capecitabine group. Also, relapse within the CNS showed a correspondingly positive trend in favor of the combination with 11 and 4 events, respectively. A median survival has not been reached yet *(114)*.

With the potential of lapatinib to cross the blood–brain barrier, treatment and prevention of brain metastasis has been of particular interest. At the ASCO meeting 2006 a study of lapatinib in MBC patients was observed. All patients had progressive or newly developed brain metastases whilst they received trastuzumab. A trend towards a benefit in the lapatinib arm was observed without reaching statistical significance *(120)*.

In conclusion, lapatinib has shown potential for the treatment of metastatic erb2-overexpressing breast cancer and investigations of the efficacy in the adjuvant setting are in progress.

7. SMALL MOLECULES IN COLON CANCER

The relevance of the family of HER receptors has been investigated in colon cancer since more than a decade. This corresponded with an interest in small molecules with inhibitory capacity against those TKs.

EGFR (HER1)mediates its downstream activity mainly via the Ras/Raf/MAPK/MAPKK (MAK kinase) *(121)* and PI3K pathways *(122,123)*. Overexpression of the EGFR receptor and its ligand have been linked to autocrine and paracrine proliferation of CRC cells as well as metastasis development and angiogenesis *(124)*. Evolving evidence suggests that constitutive activation of the HER receptors is observed during nutritional and other homeostatic stress situation *(125,126)*. This phenomenon has been implied in the malignant behavior of colon cancer cells *(127)*. Increased expression of HER2 has been observed in CRC cells and linked to a higher stage at presentation *(128)*. Signaling through EGFR/ErbB2 heterodimers has been observed after malignant transformation in colon cancer cells *(129,130)*.

The preclinical evidence described above led to clinical exploration of EGFR TKIs, mainly gefitinib and erlotinib. Both agents have been evaluated for single-agent therapy *(129,130)*. A Canadian phase II trial *(129)* evaluated erlotinib in 38 patients with pretreated metastatic CRC. The dosing schedule was erlotinib at a continuous daily oral dose of 150 mg. Of 31 evaluable patients, 19 (61%) had progressive disease and 12 (39%) had SD. In a trial exploring the single-agent efficacy of gefitinib *(130)* in pretreated, progressive mCRC, 115 patients were randomly assigned to receive gefitinib 250 or 500 mg orally once a day. One hundred and ten patients were assessable for clinical efficacy. Median FPS was 1.9 months and 4-month PFS rate was 13%. One PR was observed. Median survival was 6.3 months (95% CI, 5.1–8.2 months).

In spite of the disappointing results of both EGFR TKIs when used as single agents, both have been combined with conventional chemotherapy regimens in several phase II trials. In the first trial, 27 patients were enrolled. All participants had either progressed on a prior chemotherapy not including oxaliplatin or were intolerant to oxaliplatin. The predominant part of the patients had prior CPT-11 exposure (74%). FOLFOX-4 was administered in conjunction with gefitinib (500 mg/day) administered orally throughout the 14-day cycle in a single-armed trial design. Nine of the 27 patients (33%) had a PR by RECIST criteria. Median OS was 12.0 months. Median event-free survival was 5.4 months. The most frequently observed grade 3–4 toxicities included neutropenia (48%), diarrhea (48%), nausea (22%), and vomiting (15%) *(131)*. Another trial *(132)*, which was just reported in abstract form, enrolled 13 patients. The study regimen was gefitinib (250 mg/day) continuously from day 1, irinotecan (180 mg/m^2) as a 90-min infusion day 1, leucovorin (400 mg/m^2) over 2 h, 5-FU (400 mg/m^2) bolus, and 5-FU (600 mg/m^2) as a 22-h infusion on days 1 and 2. Cycles were repeated every 2 weeks. Dose reductions of irinotecan were reserved for patients with repeated neatropenias. Of patients receiving two or more cycles, there were 1 PR (5 months) and 6 SD (median 4 months) *(132)*. Erlotinib was combined with capecitabine and oxaliplatin in a CAPOX-type regimen *(133)*. Thirty-two patients who progressed on first-line systemic chemotherapy or had disease recurrence within 1 year of adjuvant therapy for early-stage disease. Each 21-day cycle consisted of daily oral erlotinib at 150 mg, oral capecitabine at 1,000 mg/m^2 which was reduced to 750 mg/m^2 after the first 13 patients twice daily on days 1–14 and intravenous oxaliplatin at 130 mg/m^2 on day 1 of each cycle. The median PFS was 5.4 months and the median OS was 14.7 months *(133)*. In conclusion, although the oral EGFR TKIs gefitinib and erlotinib have only shown minimal activity in CRC when used as single agents, combination with conventional chemotherapy shows promise *(134)*. This demands further study in randomized phase III trials.

Promising future perspective showed also preclinical evidence that provided efficacy on cell lines when dual HER1/HER2 inhibition was exerted *(135,136)*. Also the combination of EGFR inhibition and VEGF blockade by the small-molecule TKI showed promising preclinical results *(137)*.

8. CONCLUSION

In conclusion, this review was focused on small molecules that exercise their targeting activity below the cell surface and affect the signaling pathways mediated by TK. Our focus has been to review the current status of TKIs that are under intense investigation in a variety of phase I/II trials in patients with cancers of the breast, lung, kidney, and GI tract. Numerous previous preclinical and clinical studies have secured the role of MOABs that mediate their action on major cell-surface receptors such as EGFR *(1–4)*, also designated HER *(1–4)* family, and VEFG family *(1,2)*. The combination therapy of cytotoxic drugs with the MOAB biologic inhibitors has shown dramatic antitumor effects especially in advanced GI cancers (metastatic

CRC) in which the addition of the biological inhibitors has reinitiated an objective response to a cytotoxic drug (or drug combinations to which the tumor was previously resistant). This was accompanied by a parallel increase in PFS. This effect has been demonstrated both with the MOABs to EGFR and VEGFR. Combinations of the anti-EGFR MOAB Erbitux with radiation has also been shown to significantly enhance PFS and OS in patients with stage III/IV head and neck squamous cell cancer.

The exact role of the small-molecule inhibitors whether of TK class or other mediated pathways in cancer therapy is being defined. The potential to combine with or supplement the biologic effects of MOAB inhibitors also remains to be examined.

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VIII Impact of Nodal Status and Tumor Burden in Sentinel Nodes on the Clinical Outcome

28 Micrometastasis of Melanoma to Sentinel Lymph Nodes

Stanley P.L. Leong, MD, FACS

Contents

ABSTRACT

Micrometastasis of melanoma to sentinel lymph nodes (SLNs) is consistent with the spectrum theory of cancer progression in that cancer cells spread in an orderly fashion from the primary site to the regional SLNs in most of the time, where additional proliferation results in further spread of cancer cells to the non-SLNs and then becoming disseminated. It is important to define the extent of tumor burden in the SLNs being associated with further progression to non-SLNs and distant sites so that more rational therapy may be tailored to patients according to the tumor burden in the SLNs.

Key Words: melanoma; micrometastasis; sentinel lymph nodes

1. INTRODUCTION

Metastatic cancer is a major challenge to clinicians as effective treatment is limited. Lymph node status is the most important prognostic indicator of clinical outcome in human solid cancers. The role of the sentinel lymph node (SLN) in the process of lymphatic metastasis has been brought to the forefront. The SLN procedure has allowed us to study micrometastasis in the draining lymph

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nodes. Clinical follow-up has shown that about 80% of metastasis follows an orderly pattern of progression via the lymphatic network. On the other hand, about 20% of the time, systemic metastasis may occur either through the vascular sytems or via the lymphovenular channels.

2. SIGNIFICANCE OF MICROMETASTASIS IN MELANOMA SLNs

We and others have found that melanoma patients with micrometastasis to the SLNs have a much poorer clinical outcome *(1)*. Table 1 summarizes the studies showing that the SLN status is an excellent prognosticator for melanoma patients. The final version of the American Joint Commission on Cancer (AJCC) staging system for cutaneous melanoma by Balch et al. *(2)* defined micrometastasis as occult metastasis being detected from selective sentinel or elective lymphadenectomy. On the other hand, macrometastasis is defined from detection of gross nodal disease from therapeutic lymphadenectomy. In general, in the SLN era, it is accepted that a tumor of 2 mm or less represents a micrometastasis and any tumor over 2 mm is considered to be macrometastasis. The number of

Table 1
Summary of the clinical outcomes of melanoma patients undergoing selective sentinel lymphadenectomy. ELND = Elective lymph node dissection

Author, year	*N*	*Median follow-up*	*Outcomes*	*Significant factors*
Gadd et al. *(30)*, 1999	89 SLN –	23 months	12% recurrence	None (small numbers)
Gershenwald et al. *(31)*, 1999	85 SLN + 495 SLN –	40 months	3-year disease-free survival SLN + 55.8% 3-year disease-free survival SLN – 88.5% 3-year survival SLN + 69.9% 3-year survival SLN – 96.8%	Tumor thickness, Clark level > III, ulceration, and SLN status Tumor thickness, Clark level > III, and SLN status
Essner et al. *(32)*, 1999	42 SLN + 225 SLN – 22 ELND + 235 ELND –	45 months SLN 169 months ELND	5-year survival SLN + 64% 5-year survival ELND + 45% SLN + 38% recurrence ELND + 57% recurrence SLN – 11.5 % recurrence ELND – 14.9% recurrence	N/A because matched study for age, site, and tumor thickness
Clary et al. *(33)*, 2000	31 SLN + 121 SLN – 44 ELND + 285 ELND –	26 months SLN 79 months ELND	3-year disease-free survival SLN 80%	SLN: tumor thickness and age ELND: tumor thickness, ulceration, and Clark level

(*Continued*)

Table 1
(Continued)

Author, year	*N*	*Median follow-up*	*Outcomes*	*Significant factors*
			3-year disease-free survival ELND 71%	
Cherpelis et al. *(34)*, 2000	51 SLN + 150 SLN – (all >3.0 mm)	51 months	3-year disease-free survival SLN + 37% 3-year disease-free survival SLN – 73% 3-year survival SLN + 70% 3-year survival SLN – 82%	Ulceration Age, tumor thickness, Clark level and ulceration
Statius Muller et al. *(35)*, 2001	52 SLN + 211 SLN –	48 months	3-year disease-free survival SLN + 79% 3-year disease-free survival SLN – 95% 5-year survival SLN + 49% 5-year survival SLN – 91%	SLN status, tumor thickness, ulceration, lymphatic invasion, and age
Leong *(1)*, 2004	65 SLN + 297 SLN –	58 months	5-year disease-free survival SLN + 38.1% 5-year disease-free survival SLN – 68.6% 5-year survival SLN + 59.9% 5-year survival SLN – 68.6%	SLN status, tumor thickness, age and gender SLN status, tumor thickness, ulceration, lymphatic invasion, and mitotic index

Source: Leong SP et al. *(1)*. Reprinted with permission.

positive SLNs has also been shown to predict poor prognosis *(1)*. Although nodal metastasis is the most powerful predictor of clinical outcome in melanoma, the exact tumor burden in SLNs to predict non-SLN involvement and clinical outcome has yet to be defined. The amount of SLN micrometastasis may explain the heterogeneous clinical outcome in patients with positive SLNs.

3. FURTHER DEFINITION OF MICROMETASTASIS IN MELANOMA SLNs

In general, the tumor burden of the SLNs can be classified by hematoxylin and eosin (H&E) staining, immunohistochemistry, and reverse transcriptase polymerase chain reaction (RT-PCR) positivity. Using H&E and immunochemical staining with several monoclonal antibodies against S100, MART-1, HMB-45, melanin A, and tyrosinase, the detection ranges from 15% to 25 % by

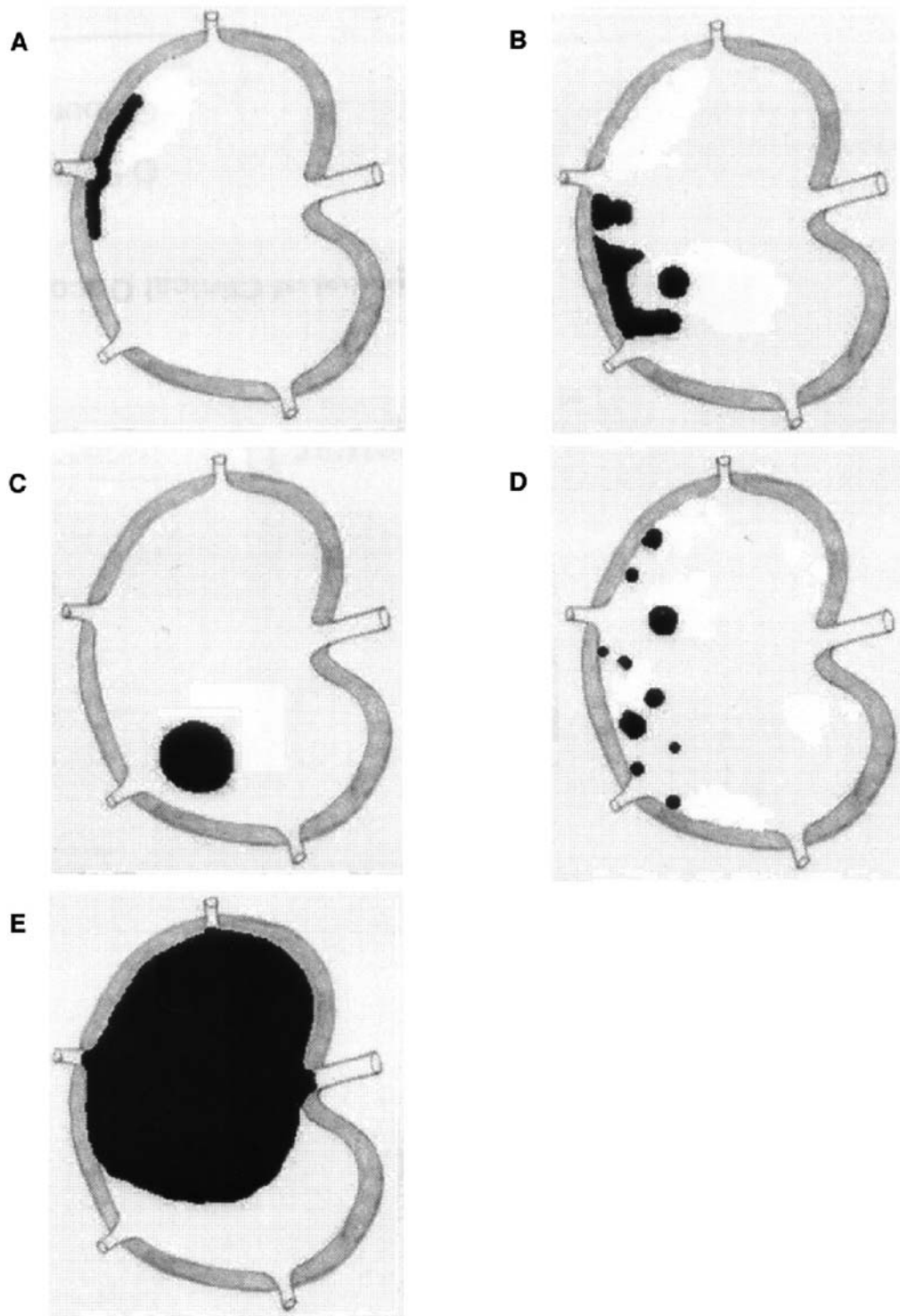

Fig. 1. Different patterns of micrometastasis in melanoma SLNs: (**A**) subcapsular, (**B**) combined, (**C**) parenchymal, (**D**) multifocal, and (**E**) extensive. (Source: Dewar et al. (2004) *(5)*. Reprinted with permission.)

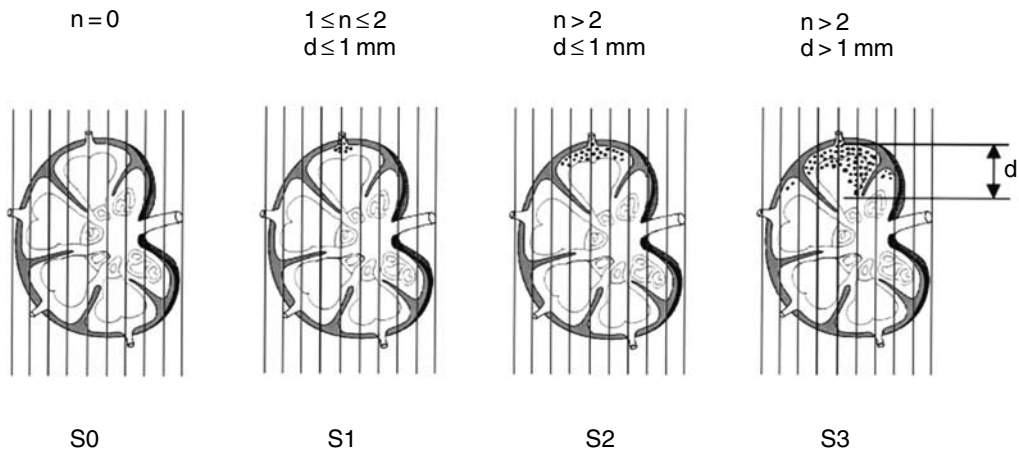

Fig. 2. Scheme of S-staging concept with 4 S classifications based on the parameters n = the number of 1-mm-thin SLN slices with immune and histologically detectable melanoma cells and d = the maximun between the distance of melanoma cells to the interior margin of the lymph node capsule. (Source: Startz et al. *[11]*. Reprinted with permission.)

Cochran/Morton protocol *(3)* and up to 18–35% by Cook EORTC protocol *(4)*. Dewar et al. *(5)* studied the correlation between patterns of micrometastasis in SLNs (Fig. 1) including subcapsular, combined subcapsular and parenchymal, parenchymal, multifocal or extensive, and the positivity of non-SLNs in the completion lymph node specimens. Patients with the subcapsular metastatic deposit (26% of a total of 146 patients) showed no involvement of the non-SLNs in the completion lymph node specimens. A recent study has also shown, by multivariate logistic regression analysis, that large SLN metastatic focus, large SLN tumor area, extracapsular extension, and synchronous deposits in the subcapsular and intramedullary areas have a strong association with positivity in the non-SLNs from the completion lymph node dissection *(6)*. Molecular staging using a single marker *(7)* and multiple markers such as tyrosinase, MART-1, gp100, and MAGE-3 can upstage patients with negative H&E and immunohistochemistry *(8)*. Because RT-PCR is a specialized technique, it is not standardized in general but more limited to specialized laboratories. Potential false-positive results may result from capsular nevus cells present up to 22% of the SLNs *(9)*. For this reason, frozen sections of SLNs are usually not used in melanoma to determine positivity for intraoperative decision to proceed with a formal regional lymph node dissection *(10)*.

Further, the depth of nodal invasion by Starz et al. *(11, 12)* (Fig. 2) has been correlated with clinical outcomes in 281 patients with positive SLNs. S0 represents a negative SLN. The distance of the metastasis deposit between the edge of the SLN and the innermost margin is represented by SI, SII, and SIII, with SI being the shortest. The S-classification is highly reliable with the prediction of positivity of non-SLNs and the clinical outcome with S0 and SI being the most favorable groups. Other studies have also shown the clinical significance of micrometastasis in melanoma SLNs *(13–17)*.

4. DATA FROM RANDOMIZED STUDIES TO SHOW THE SIGNIFICANCE OF MICROMETASTASIS IN MELANOMA SLNs

In two recent prospective randomized studies, micrometastasis in SLNs has been shown to be clinically significant. A total of 1,269 patients with primary cutaneous melanoma were randomized to wide excision and observation with lymph node dissection of the nodal basin if recurrence of the basin occurred versus wide excision and selective sentinel lymphadenectomy

followed by completion lymph node dissection if the SLNs were positive *(18)*. The SLN status was found to be an excellent prognosticator for clinical outcome with the SLN-negative group to be faring much better than patients with positive SLN(s). Further, for patients with primary of melanomas ranging from 1.2 to 3.5 mm, those with immediate lymphadenectomy for a positive SLN with micrometastasis have a much better survival than those when lymphadenectomy was performed for grossly palpable disease in the regional nodal basin.

According to the Rotterdam criteria for SLN tumor burden, the maximum diameter of the largest lesion of a positive SLN is recorded as the tumor burden measurement. If multiple positive SLNs are present, the maximum diameter of the largest lesion of all positive SLNs represents the largest dimension overall. Using this definition, a total of 388 melanoma patients with positive SLNs from three European centers were analyzed with a median follow-up of 36 months. The three groups of patients included (1) submicrometastasis with at least 10 cells but less than 0.1 mm, (2) micrometastasis between 0.1 and 1.0 mm, and (3) micrometastasis greater than 1.0 mm. The distribution of these three groups were 10, 35, and 55%. They have found that there were no two or more SLNs with submicrometastasis. Submicrometastsis occurred in every T-stage, and patients with submicrometastasis had an excellent survival, being identical to SLN-negative group. Tumor burden in SLNs increased with T-stage. Both T4 and SLN tumor burden were the most important factors for survival *(19)*. In a separate study, 1,256 melanoma patients with stage III disease were randomized to pegylated interferon versus observation. Randomization was stratified for nodal involvement N1 (microscopic) versus N2 (palpable nodes) and other high-risk factors. With a median follow-up of 3.8 years, patients with only microscopic nodal involvement or SLN positivity seemed to have a greater benefit with respect to both relapse-free survival and distant metastasis-free survival *(20)*.

5. RECENT DATA ON MICROMETASTASIS IN MELANOMA SLNs FROM UCSF

Recently at UCSF Helen Diller family Comprehensive Cancer Center we have completed a study analyzing a total of 63 melanoma patients (41 males/22 females) with micrometastasis in the SLN(s) following selective sentinel lymphadenectomy from 1994 to 2003 with 7.8 years of follow-up. The SLN metastasis was microscopically assessed for size, number of foci, and anatomic location (subcapsular sinus, parenchymal, and sinusoidal) by H&E. In multivariate analysis, after adjusting for age and gender, the maximum metastatic deposit (greater than 1 mm) and primary thickness were the most important prognostic factors for disease-free survival ($p =$ 0.05 and 0.01) and overall survival ($p =$ 0.009 and <0.0001), respectively. Larger multicenter database may be required to determine the optimal maximum metastatic deposit cutpoint *(21)*.

6. NEW PARADIGM OF METASTASIS FOR MELANOMA BASED ON THE SLN DATA

Based on these studies, the SLN data have emerged to show that melanoma metastasis is a progressive process with initial transformation of melanocytes, through melanogenesis to become melanoma in situ. Further proliferation may result in invasion of melanoma through the basement membrane with increasing thickness, which may spread to the SLN and beyond (Fig. 3) *(22, 23)* via the lymphovascular system *(24, 25)*. In general, melanoma progresses from in situ growth to a radial growth phase and then to a vertical growth phase, which is associated with increased risk of metastasis. Breslow tumor thickness as measured microscopically from the stratum granulosum of the epidermis to the deepest point of the primary melanoma is the best predictor of clinical outcome. It

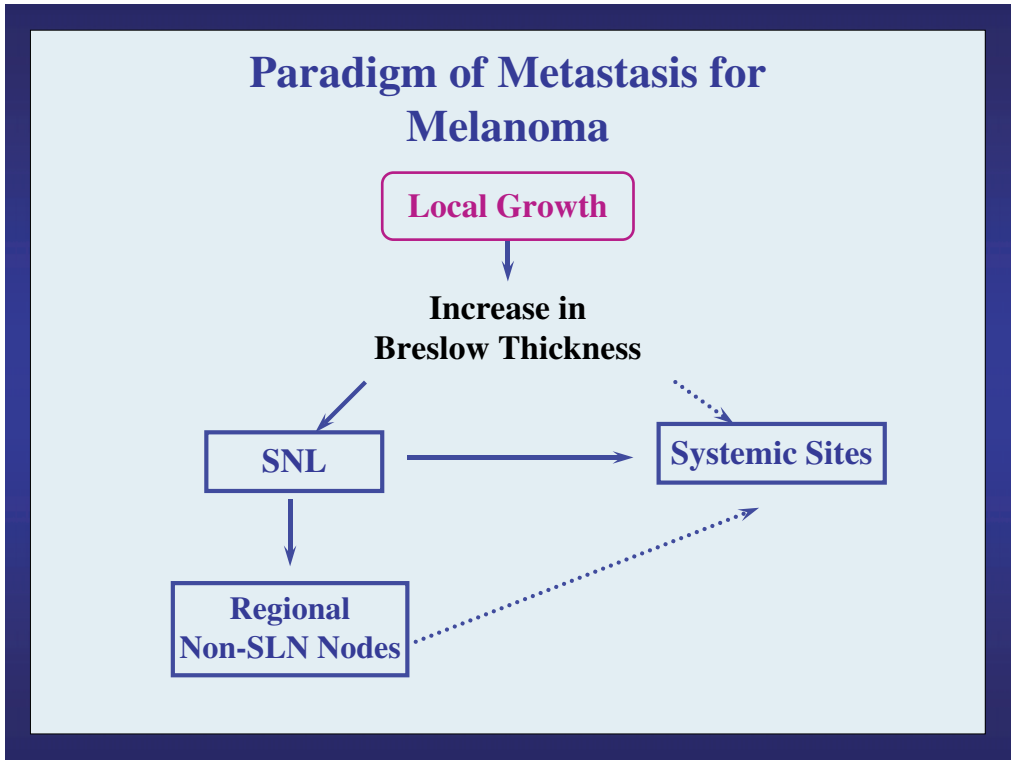

Fig. 3. Orderly progression of invasive melanoma to the SLN, non-SLN, and then the distant sites. Occasionally, melanoma bypasses the SLN system and spreads to the distant sites.

is an integral part of a melanoma pathology report *(26)*. It has been repeatedly emphasized in this chapter that regional nodal status correlates significantly with survival. When metastasis is found beyond the regional lymph nodes, especially in visceral sites, the survival rate drops to single digits *(2)*.

Micrometastasis may include a single cell, cluster of cells, <0.1 mm, 0.1–2 mm, greater, and beyond. The clinical significance of each subgroup of patients with these findings remains to be defined as such data were not even available for the sixth edition of AJCC staging of melanoma *(2)*. It has been suggested that micrometastasis is biologically different from macrometastasis *(27)*. Certainly, macrometastis is more aggressive and ominous. Resection of micrometastasis may yield a better clinical outcome. Thus, the melanoma metastasis data in the SLN era are consistent with the spectrum theory of cancer progression *(28, 29)*.

7. CONCLUSION

The spectrum hypothesis of cancer progression *(28, 29)* forms the current paradigm of metastasis for melanoma. Therefore, most of the time, early melanoma, when resected, may result in the cure of patients.

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29 Sentinel Lymph Node Micrometastases in Breast Cancer: Prognostic Relevance and Therapeutic Implications

Igor Langer, MD, Ulrich Guller, MD, MHS, Carsten Thomas Viehl, MD, and Markus Zuber, MD

CONTENTS

ABSTRACT

OBJECTIVE

The objective of this chapter is to provide a comprehensive overview of the impact of sentinel lymph node (SLN) micrometastases (>0.2 to ≤ 2.0mm) on axillary recurrence rate and overall survival (OS) in breast cancer patients.

INTRODUCTION

The SLN procedure proved to be a reliable method for the evaluation of the axillary nodal status in patients with early-stage invasive breast cancer. Therefore, level l and II axillary lymph node dissection (ALND) can be omitted if the SLN is free of macrometastases. However, the

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clinical relevance and therapeutic implications of SLN micrometastases remain a matter of great debate. Herein, we present data from our own prospective SLN study, which will be discussed in the context of other published investigations particularly focusing on the axillary recurrence rate and OS in patients with SLN micrometastases.

CONCLUSION

Based on current literature and our own study results it can be concluded that there is no difference regarding axillary recurrences in patients with SLN micrometastases after SLN biopsy alone compared with either SLN-negative patients or breast cancer patients undergoing a formal ALND. In our investigation there was no statistically significant difference in OS, rate of axillary recurrences, and distant disease-free survival between patients with negative SLN and those with SLN micrometastases, who did not undergo a completion ALND. Despite a false-negative rate of the SLN procedure of about 10% as well as a substantial proportion of positive non-SLN lymph nodes in patients with SLN micrometastases, there appears to be little clinical impact of these phenomena. In fact, most SLN micrometastases do not negatively impact regional and distant disease control.

Key Words: breast cancer; sentinel node; micrometastases; axillary recurrence; survival

1. INTRODUCTION

The axillary lymph node status is among the most important prognostic factors in breast cancer patients and helps defining the need for subsequent adjuvant treatment. Over the past decade, the sentinel lymph node (SLN) biopsy has emerged as the standard staging method for the axillary lymph node status in early-stage breast cancer patients in most institutions and has replaced standard level I and II axillary lymph node dissection (ALND). Many studies demonstrated the accuracy and the high negative predictive value of the SLN procedure *(1)*. Therefore, node-negative patients can be spared the substantial short- and long-term morbidity of a level I and II ALND *(2)*.

One of the main advantages of the SLN biopsy is that the pathologist can focus his or her attention on a few lymph nodes only *(3)*. This permits the systematic use of step sectioning and immunohistochemistry (IHC) in the analysis of the SLN, which would not be feasible in the assessment of all nodes of an ALND specimen due to time and financial constraints. The systematic use of step sectioning and IHC for SLN analyses results in a higher detection rate of micrometastases. However, the clinical relevance of SLN micrometastases in terms of long-term disease-free survival (DFS) and overall survival (OS) and the therapeutic implications regarding a completion level I and II ALND remain controversial. The objective of this chapter is to discuss the prognostic impact and therapeutic relevance of SLN micrometastases in breast cancer patients. Prospective data from our own institution will be discussed in the context of current relevant literature.

2. ACCURACY OF THE SLN PROCEDURE AND AXILLARY RECURRENCE RATE AFTER SLN BIOPSY ALONE

Numerous studies demonstrated the accuracy and the high negative predictive value of the SLN procedure *(1,4)*. However, the SLN procedure is associated with a false-negative rate of 8–10% when summarizing larger series *(5,6)*. Krag et al. recently published the NSABP B-32 trial data including 5,611 patients. The false-negative rate of the SLN procedure was 9.8% *(7)*.

Nevertheless, the axillary recurrences are very low with reported rates ranging from 0 to 2% even during long-term follow-up *(5,8,9)*. Two randomized trials revealed no significant difference of axillary recurrences when comparing node-negative patients undergoing SLN only versus level I and II ALND *(5,10)*. This implies that SLN biopsy not only provides accurate staging but also excellent regional control of clinically occult disease. Veronesi et al. demonstrated in their study of 953 SLN-negative patients who did not undergo a completion level I and II ALND that the incidence of axillary recurrence (0.3%) after a median follow-up of 38 months was much lower than expected *(11)*. It appears that some microscopic foci in axillary lymph nodes will never progress into clinically relevant metastases. In fact, experimental studies suggest that most isolated tumor cells are not viable and will not progress into clinically apparent disease *(12,13)*. A variety of biological tumor cell characteristics such as viability, angiogenic capacity, and avoidance of the host immune reaction may play an important role in the progression from isolated tumor cells to clinically relevant metastatic disease. Furthermore, adjuvant chemotherapy also contributes to the locoregional control. In fact, nodal tumor infiltrates were found to be sensitive to various chemotherapeutic agents, which resulted in the ablation of residual microscopic axillary metastases *(14)*.

3. DETECTION RATE OF SLN MICROMETASTASES

Since the introduction of the SLN procedure into clinical practice, axillary lymph node micrometastases are more frequently detected *(4,15)* as pathologists can focus on a few lymph nodes only. Step sectioning and IHC can be routinely applied for the analysis of SLN *(16)*. Conversely, the systematic use of such techniques is not feasible in the assessment of all nodes of ALND specimens, as these procedures are time consuming and costly. The detection rate of micrometastases varies according to different histopathological techniques and protocols. Current literature reports that 15–48% of all SLN tumor deposits are micrometastases, leading to an upstaging of node-negative patients in 9–25% *(15–17)*.

In an investigation from our institution, SLN biopsy was performed on 224 early-stage breast cancer patients *(8)*. The SLN were tumor-free in 123/224 patients (54.9%), contained macrometastases in 74/224 (33.0%), and micrometastases in 27/224 patients (12.1%). Micrometastases were detected by step sectioning with hematoxylin and eosin (H&E) staining in 14/27 cases and by the use of additional IHC in 13/27 patients. The identification of micrometastases led to a formal upstaging in 18% (27/150) of node-negative patients.

4. CORRELATION OF SLN AND NON-SLN METASTASES

Several studies examined the correlation of SLN and non-SLN metastases *(18–20)*. Some investigations, in which standard histological examinations of non-SLNs (bisection, H&E staining) were performed, reported rates of non-SLN metastases ranging from 0 to 30% in patients with SLN micrometastases *(19,21–23)*. Another investigation found that the percentage of detected metastases in the non-SLN lymph nodes increased up to 53% with the use of step sectioning and IHC in the ALND specimen as compared with standard pathology protocols *(19)*. Although the pathology techniques were variable among different studies, a summary revealed an overall non-SLN involvement of 18.6% (range 0–35%) in patients with SLN micrometastases *(3,21,24–27)*. Interestingly, up to 26% of patients with isolated tumor cells in the SLN show a non-SLN involvement *(3,21,24)*. In multivariable analysis, large size of the primary tumor or SLN micrometastases, presence of peritumoral lymphatic invasion, and number of involved SLN were significantly associated with the presence of non-SLN metastases

in patients with SLN micrometastases *(3,16,24)*. Moreover, patients with SLN micrometastases detected by H&E staining and step sections were more likely to harbor further non-SLN metastases as compared to patients with SLN micrometastases detected by IHC *(16,18,24,26)*. In deed, patients with SLN micrometastases detected by H&E staining and step sections had further non-SLN metastases in the ALND specimens in 18.5% (range 8.5–36.3%), whereas non-SLN metastases in patients with IHC-detected SLN micrometastases occurred in 11.5% (range 0–28.6%) *(16,24,26,28)*.

5. TYPE OF NON-SLN METASTASES IN PATIENTS WITH SLN MICRO-METASTASES

Different studies analyzed the type of non-SLN metastases *(3,21–23,27,29)*. For both subsets of patients, those with SLN isolated tumor cells as well as those with SLN micrometastases, almost all studies showed that the vast majority of non-SLN metastases were macrometastases. Patients with SLN micrometastases and positive nodes in the completion ALND specimen were found to have non-SLN macrometastases in 60%, non-SLN micrometastases in 28%, and non-SLN isolated tumor cells in 12% *(3,16,21,23)*. Patients with isolated tumor cells in the SLN and non-SLN involvement had macrometastases in 72%, micrometastases in 19%, and isolated tumor cells in 9% of the examined ALND specimens *(3,21,29)*.

Although it was shown that most breast cancer patients undergo adjuvant systemic therapy regardless of the axillary node status, some patients would be undertreated, if a completion ALND were not performed for SLN isolated tumor cells or micrometastases *(30)*. Chagpar et al. detected micrometastases in 18% of previously node-negative SLN by systematically adding step sections and IHC to the initial histopathological analysis *(30)*. They reported that the detection of micrometastases resulted in a management change in 13% of patients. In another study by van Rijk et al., 7% of patients with SLN micrometastases, but no patients with SLN isolated tumor cells would have been undertreated, if a completion ALND were not performed *(21)*. In their study, undertreatment would have occurred in only 0.4% of all patients.

6. PROGNOSTIC SIGNIFICANCE OF SLN MICROMETASTASES

The prognostic and therapeutic implications of SLN micrometastases remain a matter of great debate. Unidentified micrometastases have been held responsible for the occurrence of up to 30% distant metastases of breast cancer patients with negative axillary lymph nodes after ALND *(31)*. Some of these patients might benefit from adjuvant therapy. In the ALND era various retrospective studies reported a DFS and OS disadvantage in breast cancer patients with micrometastases *(32)*, others, however, failed to find any significant association *(33)*. In a recent, retrospective study, Colleoni et al. analyzed the impact of minimal node involvement on survival in 1,959 breast cancer patients *(34)*. An SLN biopsy was performed in only 43% of patients. After a median follow-up of 49.9 months patients with minimal lymph node involvement, defined as the presence of micrometastases or isolated tumor cells, had a worse 4-year DFS compared with node-negative patients. However, there was no difference in OS. In the SLN era, Hansen et al. reported a 5-year DFS of 98.3 and 94.5% for patients with SLN micrometastases detected by IHC and H&E step sections, respectively. The 5-year OS was 100% in each group (median follow-up 38 months) *(35)*. There was no significant DFS or OS difference between patients with SLN micrometastases and SLN-negative patients ($n = 425$) (DFS = 95.1%, OS = 99.7%). However, all patients with SLN micrometastases underwent a completion ALND. Similarly,

Veronesi et al. compared SLN-negative patients with patients with SLN micrometastases undergoing completion ALND and found no difference in the rate of breast cancer-related events *(11)*.

In an investigation from our institution, DFS and OS was compared between 123 node-negative breast cancer patients and 27 patients with SLN micrometastases *(8)*. None of these patients underwent a completion level I and II ALND. In the SLN-negative group, 4.9% of the patients (6/123) recurred after a median follow-up of 42 months. Two local (2/123, 1.6%) and one axillary recurrence was observed (1/123, 0.8%), whereas distant metastases were identified in three patients (3/123, 2.4%) during follow-up. Conversely, in the SLN micrometastases group no local, axillary, or distant disease recurrence were observed.

There was no statistically significant difference for distant and axillary DFS ($p = 0.15$) and OS ($p = 0.66$) between SLN-negative patients and those with SLN micrometastases. Our prospective investigation provides suggestive evidence that a completion ALND can be safely omitted in patients with SLN micrometastases sparing the substantial morbidity of an ALND.

Our study represents the only published series of an unselected group of patients with SLN micrometastases, in whom a completion ALND was systematically omitted *(8)*. In nearly all other publications reporting patients with SLN macrometastases, micrometastases, or isolated tumor cells, in whom a completion ALND was omitted, the patient selection was based on various criteria and therefore potentially biased: patients refusing further surgery, elderly patients, frail and comorbid patients, or patients felt to be at low risk for having residual axillary disease according to the nomogram from the Memorial Sloan Kettering Cancer Center (MSKCC) *(36–39)*. Interestingly, in a previously mentioned investigation, Chagpar et al., reviewed patients with negative SLN status determined by standard H&E staining of bivalved sentinel nodes who did not undergo a completion ALND *(30)*. The SLN specimens were reevaluated by step sections and IHC. A total of 12 out of 84 patients were identified as having SLN micrometastases. However, no distant metastases or deaths were reported in this group after a median follow-up of 40 months. No statistically significant distant DFS and OS was observed between the patients with negative SLN and those having SLN micrometastases *(30)*. Regardless of the fact that most studies examined selected patient groups, only few recurrences were observed in patients with SLN micrometastases or SLN isolated tumor cells after follow-ups ranging from 12 and 81 months *(30)*. Furthermore, even patients with SLN macrometastases who did not undergo further axillary surgery had an excellent regional and distant disease control after follow-ups between 25 and 48 months *(37,39–41)*. However, most of these patients were at low risk of having further positive lymph nodes in the axilla according to the MSKCC nomogram and may have also benefited from adjuvant chemotherapy.

7. CONCLUSION

In summary, axillary recurrences in patients with SLN micrometastases do not occur more frequently after SLN biopsy alone compared with either SLN-negative patients or breast cancer patients undergoing a formal level I and II ALND. Our own data are in concordance with these findings. In fact, there was no statistically significant difference in OS, rate of axillary recurrences, and distant DFS between patients with negative SLN and those with SLN micrometastases, who did not undergo a completion ALND. Despite a false-negative rate of the SLN procedure of about 10% as well as a substantial proportion of positive non-SLN lymph nodes in patients with SLN micrometastases, these phenomena appear to have little clinical relevance. In fact, most SLN micrometastases do not negatively impact regional and distant disease control.

This could be explained by the fact that almost all breast cancer patients will undergo some adjuvant treatment. Moreover, not all disseminated tumor cells have the capacity to progress into clinically apparent disease.

The current literature as well as our investigation provide suggestive evidence that a completion ALND can be safely omitted in patients with SLN micrometastases. These patients can be spared the substantial short- and long-term morbidity of a formal level I and II ALND. Ongoing prospective trials from the National Surgical Adjuvant Breast and Bowel Project (NSABP) and International Breast Cancer Study Group (IBCSG) are hoped to provide a definitive answer regarding prognostic and therapeutic implications of micrometastases in breast cancer patients.

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30 Micrometastasis of Genitourinary Cancer to Sentinel Lymph Nodes

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CONTENTS

ABSTRACT

A majority of the more frequent tumors can expand through the lymphatic system, affecting progressively an increasing number of lymph nodes (LNs).

LN status is among the most important prognostic indicators for the clinical outcome of patients with solid malignancy. With the advent of the application of the sentinel lymph node (SLN) concept, significant advances have been made but new unanswered questions exist. The finding and understanding of micro- and submicrometastases is an interesting biological phenomenon, which has been investigated and the final result still is far away.

Therefore worldwide cooperative study in "micrometastasis" between national and international investigators is necessary for its definitive validation.

Key Words: sentinel lymph nodes; micrometastasis; natural history of testicular carcinoma metastasis; recent developments of dynamic SLN in urology

1. INTRODUCTION

The presence of metastasis in the sentinel lymph node (SLN) defines malignancy. The application of the concept of the SLN of any solid tumor preferentially should be in early stage, clinically negative lymph node (LN) *(10,11,42)*.

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From the surgical point of view, by physical examination of the lymphatic system, the metastatic LN could be local, regional, or distant metastases, "in transit" and "interval" belonging to malignant melanoma. The concept of micrometastasis and macrometastases was proposed in 1971 by Huvos et al. *(28)* but its importance grew up in the 1990s after the advent of the sentinel lymph node biopsy (SLNB) technique, and was incorporated to the tumor–node–metastasis (TNM) system later on *(26,74)*.

Micro- and submicrometastases were defined at the Philadelphia Consensus Conference in 2001. Metastases >0.2 mm and ≤2 mm are categorized as micrometastasis (pNiMi). Tumor deposits or isolated tumor cells in the marginal sinus ≤0.2 mm as submicrometastasis (pNo) *(4,65,66,74,77)*.

The classification of submicrometastasis was a direct consequence of the progress obtained in the immunohistochemistry stain as well as in molecular biology. Again isolated tumoral cells not >0.2 mm are classified as pNo submicrometastasis. Later on the term nanometastasis *(60)* was coined to replace the term isolated tumor cells *(26,65,66)*.

Another concept that is very important to remember is "occult metastases," which means nondetected metastases using hematoxylin–eosin, but the metastatic process was found by other techniques *(4,6,7,18,23,24,27,28)*. The question if we can applied in urological cancer, the clinical experience accumulated in breast cancer, that in a patient with "axillary SLNB disclosed micrometastasis, the completion of axillary lymph node dissection be avoided?"

All the clinical experience accumulated in SLNB in malignant melanoma *(72)* as well as breast cancer (SLN micrometastasis) are very fascinating, but not necessarily be extrapolated in urological malignancies for therapeutical purposes.

Although the sentence was given more than 10 years ago, and never was more contemporaneous, "that the demonstration of lymph node-only metastatic cells, suggests an explanation for lymph nodes metastases as indicators but not Governors for therapy" *(18)*. The concept of SLNB made unacceptable the morbidity and complications of the prophylactic LN dissection *(10,11,13–16,18,33)*.

Limitations in time and space preclude a complete review of current knowledge of the micrometastases in urology malignancies, but the existing problems will be pointed out, and the solutions shall be a duty of the next generations.

2. PENILE CARCINOMA

The results obtained in the application of the SLNB was not recognized in order to classify penile carcinoma in the TNM system. Numerous publications *(6,7,10,11,13–16,27,31,33,37–40,58,73,86)* assessed the values of the dynamic sentinel lymph node biopsy (DSLNB) with pioneers, ideas, and great refinement techniques obtaining 97% of visualization of SLN. The technical details to identify the SLN, the mechanism of the metastatic process, the protocols of the molecular investigation, and the immunohistochemical staining are beyond the scope of this chapter. Previous publications described the technique of the DSLNBs and we will not explain in detail, although we will point out the contemporaneous investigations that is going on.

Bin K. Kroon, under the tutelage of Horenblas, B.B. Kroon, Nieweg, and Valdes *(27,31,32,33,58)*, presented his thesis summarizing the experience of the Netherlands Cancer Institute reports the evaluation of the micrometastases in SLN identified using immunohistochemical staining—Pankeratin (Becton Dickinson, San Jose, CA). He concluded " The risk of non-sentinel node involvement in penile carcinoma patients is correlated with the size of the SN metastases." Groins with micrometastasis in the SLN only did not have additional metastasis in the rest of the inguinal LNs" (micrometastasis<2 mm or macrometastases >2 mm *[6,7,31,32,33]*).

Perdona et al. performed SLNB in 17 cases bilaterally in clinically node-negative patients. Five patients had a positive sentinel node (SN). In all patients, subsequent LN dissection was carried out and all negative SLNB were confirmed by tumor-negative dissection specimen *(58)*.

Recently, in an attempt to improve the accuracy of the SLNB, carbon particles can be added to the tracers as an aid to direct the pathologist to the site in the SN most likely to contain metastases *(23,77)*.

Radionuclide imaging is renowned for its capabilities in functional imaging, but it suffers from limited spatial resolution and imprecise anatomic localization, whether performed as planar imaging or a single-photon emission computed tomography (SPECT). Computerized tomography (CT) imaging excels in depicting anatomic details. The relative advantages of both modalities are now available in single hybrid devices.

The combination of lymphoscintigraphy with CT (hybrid SPECT-CT system) enables preoperative localization of SN in anatomic details and facilitates detection intraoperatively *(20,37,40,71,79–82)*.

Indications are 1) Planar lymphoscintigrams with unusual lymphatic drainage patterns. 2) Planar images that were difficult to interpret or planar lymphoscintigrams with nonvisualization. Eventually, SPECT-CT led to upstaging and change in management, offering better anatomic localization and attenuation correction *(40)*.

Magnetic resonance lymphangiography is a promising technique in the detection of occult LN metastases. This novel technique makes use of a LN-specific contrast agent that allows the identification of clinically occult metastasis. This contrast agent, known as ultrasmall particles of iron oxide (USPIO) is injected intravenously and is taken up primarily by macrophages in the LNs. Presence of USPIO in the node results in signal intensity loss (darkening) on the T2-weighted sequences. Metastatic growth will displace the macrophages filled with USPIO, which results in the metastatic part of the node being high in signal intensity (whitening). Thus a metastasis in the LN will show as a white filling defect. Metastases as small as 1 mm have been detected using this technique *(24,47,50,76,91,94)*.

In a mouse model, even as few as 1,000 tumor cells could be depicted *(94)*. Preliminary results of this technique in penile carcinoma have been reported *(40,47,94)*.

3. PROSTATE GLAND

The major advance in the evaluation of carcinoma of the prostate gland is the achievement of the visualization of the lymphatic system draining the organ; many investigators obtained the same results *(79,80,85,87,88,89,90–92)*.

^{99m}Tc-nanocolloid was administered in both lobes of the prostate guided by transrectal ultrasound. Tracer volume was injected peri/intratumorally in four depots of 0.1 ml followed each time by flushing with 0.7 cc normal saline using a three-way system. Resting injection device activity was measured to calculate net dosages. Planar scintigraphy is performed at 15 min, 2, and 4 h. SPECT-CT camera was performed at 4 h. The first appearing LNs were identified as SLN and differentiated from secondary nodes depicted on delayed images. Gamma probe guided SLN dissection followed by laparoscopic extended lymphadenectomy or by open surgery. Mean injected dosage was 122 MBq (range 123–266) with rate of 88% at 15 min and 96% at 2 and 4 h. SLN visualization rate was 96%, and 50% of SN identified outside the expected drainage stations *(48,49)*. Lymphoscintigraphy at 15 min and 2 h complemented by SPECT-CT appears to be an adequate protocol to guide laparoscopic SN location in prostate cancer. Transrectal ultrasound-assisted tracer administration enables inspection of approximately 90% of the planned doses *(79,80,85,87,88–90)*.

Recently, several authors reported their experience concerning SLNB in prostate gland, where metastasis was found in about 20% of the patients; 63% of these metastases were found in outside the region of standard pelvic lymphadenopathy (PL). With a sensitivity of 96%, SLNB appeared to be a reliable method to replace standard PL in patients of the intermediate risk group or to be combined with PL in patients of the high-risk group *(48,49,79,80,81,85,87–92)*.

Because routine microscopic examination of lymphadenectomy specimens can miss small cancer foci, this finding might partially account for the presence of histologically undetectable micrometastasis in the pelvic LNs. In fact, various investigators have shown the higher sensitivity for detecting micrometastatic cancer cells in surgically removed pelvic LNs at radical prostatectomy can be achieved by several molecular and histologic techniques targeting prostate-specific gene expressions, including reverse transcriptase PCR (RT-PCR) and immunohistochemical staining. Today however, none of these methods have been introduced into clinical practice due to various limitations such as a high false-positive rate and complicated procedures *(51,52)*.

In 1992, Moreno et al. *(51)* initially reported the clinical application of RT-PCR for detecting micrometastatic prostate cancer cells in the peripheral circulation of patients with prostate cancer. Since this report, there has been a rapid expansion of the use of RT-PCR for the detection of micrometastatic prostate cancer cells in peripheral blood, regional LNs, bone marrow, and surgical blood *(3,5,19,21,24,25,29,30,52,57,59,60,63,77,89,95)*.

In 1993, DeGuchi et al. reported the use of an RT-PCR assay targeting prostate-specific antigen (PSA) mRNA to detect micrometastatic prostate cancer cells in LNs. The assays detected PSA mRNA in five LNs with histologic and/or immunohistochemical evidence of metastatic cells and in 4 out of 30 LNs with no histologic or immunohistochemical evidence of metastasis *(17)*.

Miyake et al. *(51,52)* performed quantitative detection of micrometastasis in pelvic LNs in patients with clinically localized prostate cancer by real-time reverse transcriptase. Routine pathologic examination can miss micrometastasis tumor foci in the LNs of patients with prostate cancer. They tried to clarify the significance of micrometastasis in pelvic LNs in patients who underwent radical prostatectomy for prostate cancer. Pathologic examinations detected tumor cells in 29 LNs from 11 patients and real-time RT-PCR further identified micrometastasis in 143 LNs from 32 patients with no pathologic evidence of LN involvement. The presence of micrometastases was significantly associated with other conventional prognostic variables. They concluded that 30% of clinically localized prostate cancers shed cancer cells to the pelvic LNs and the biochemical recurrence after radical prostatectomy could be explained, in part, by micrometastases in pelvic LNs *(51)*.

Complete LN dissection reveals a high rate of metastases (25%). In patients with positive node dissection, time to progression is significantly correlated with the number of diseased nodes. Some patients with minimal metastatic disease remain free of PSA relapse for more than 10 years after prostatectomy without any adjuvant treatment. Meticulous pelvic LN dissection particularly in patients with micrometastases seems not only to be a staging procedure but may also have a positive impact on diseases progression and long-term disease-free survival *(3,19,21,24,30,51,52,58,63,95)*.

4. BLADDER CARCINOMA

Nodal status in urothelial urinary bladder carcinoma is a parameter with significant prognostic importance. Presence of nodal dissemination implies low survival figures even in 1, 2, and 5 years after definitive treatment. This, regardless of maximum surgical and oncological treatment modalities. A finding of metastatic LNs may also be indicative for further oncological attempts to control the disease in a preoperative setting.

Adjuvant chemotherapy is thus a part of the therapeutic arsenal being utilized. The standard therapy is meticulous and ambitious attempt to perform wide and extensive LN surgery, a simple viable node with a micrometastatic deposit might be remaining in the patient after surgical closure *(11,41,68,70)*. Node dissections in many centers is limited to the obturator fossa.

SN detection in urothelial urinary bladder carcinoma has shown the presence of tumor-draining nodes as well as nodal metastatic deposits beyond standard dissection areas. SLN improves the staging so that positive SN can be found outside areas removed in extended LNs dissection. Tumor-reactive lymphocytes are present in SN-draining human bladder cancer. These cells display immunological function upon restimulation in vitro *(1,3,8,35,44,70)*.

The SN concept in urothelial bladder carcinoma can be a method of improving nodal staging and refined surgical dissection and might even be a starting point for experimental methods aiming at improving survival *(69,70)*.

SN detection-based (Sentoclone) therapy utilizing a method entailing infusion of expanded antilogous tumor-specific T helper cells has been developed *(45,70)*.

Preoperative lymphoscintigraphy, preoperatory dye injection, and dynamic lymphoscintiography are available today; the substance is injected adjacent to the tumor in the detrussor muscle *(35,36,68,69)*.

In intraoperative SLN detection, nodal staging in invasive bladder cancer was feasible although the false-negative rate was 19%. Extended serial sectioning and SPECT-CT offer tremendous benefits for lymphatic mapping and tumor detection *(20,69,71,94)*.

Can immunohistochemistry enhance the detection of micrometastasis in pelvic LNs from patients with high-grade urothelial carcinoma bladder? Attempts have been made for detection of micrometastases in pelvic LNs in patients undergoing radical cystectomy for locally invasive bladder cancer by real-time RT-PCR for cytokeratin 19 and uroplakin II *(1,8,34,35,36,44,68,70)*. Immunohistochemistry revealed micrometastasis in SLN in nine patients and radioguided surgery after the completion of lymphadenectomy identified SLN metastasis in an additional seven *(2,8,35,36)*.

Approximately, 30% of locally invasive bladder cancer shed cancer cells to pelvic LNs and disease recurrence after radical cystectomy could be explained at least in part by micrometastases in pelvic LNs *(34)*.

In bladder cancer, altered E-cadherin expression is associated with the degree of invasiveness, LN metastasis, and increased risk of death from bladder cancer. Furthermore, E-cadherin status is an independent predictor of disease progress in patients treated with cystectomy for transitional cell carcinoma of the bladder *(8)*.

5. TESTICULAR CARCINOMA

The illustrations disclosed the sequences of metastasis in malignant melanoma versus testicular carcinoma. The natural history of the metastatic process until advanced stage *(10,12)* (Figs. 1–5 and Color Plates 15–18). The management of the regional LNs in Stage I testicular carcinoma is still surrounded by controversy. As a result of the low incidence of occult LN metastases, primary treatment for nonseminomatous germ cell tumors in the form of retroperitoneal lymphadenectomy or chemotherapy results in overtraatment with associated morbidity in 70–75% of the patients. Overtreatment in seminomatous germ cell tumors with routine adjuvant radiotherapy is even higher, because occult metastases are present in less than 20% of the patients *(56,64,75)*.

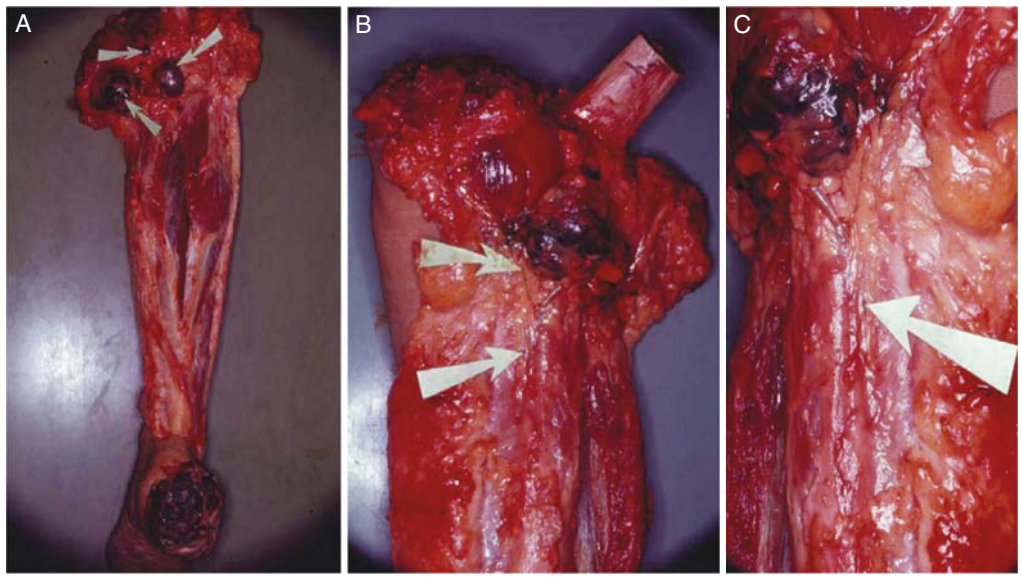

Fig. 1. (**A**) Malignant melanoma of the heel with popliteal SLN metastasis. (**B**) The cancer cells reach the regional nodes by "embolization" (*arrows*) rather than by centripetal permeation, discontinuous embolism *(10,22,62,93)*. (C)*Arrow* indicates metastatic embolus. (*see* Color Plate 15)

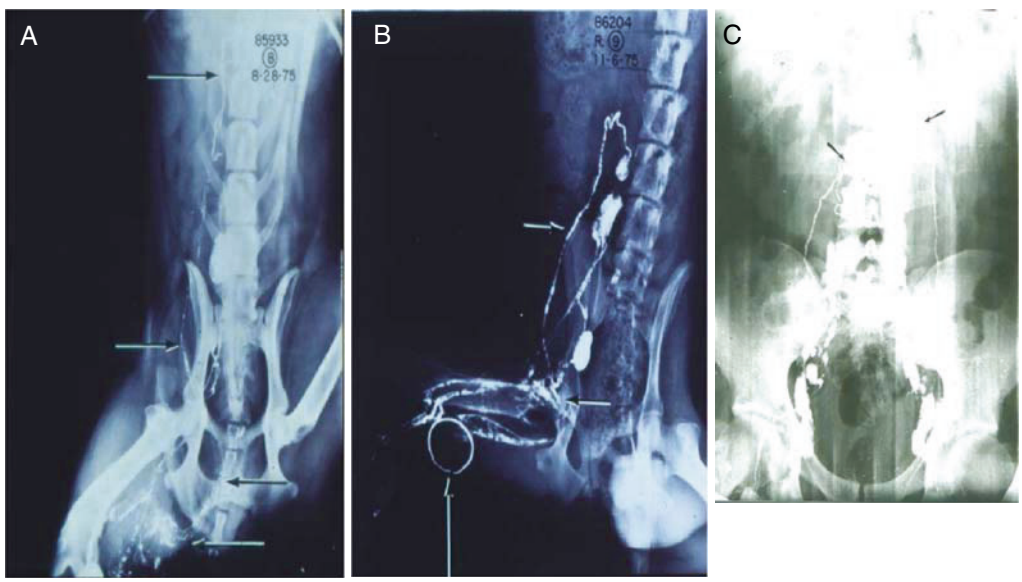

Fig. 2. (**A**) Lymphangiogram showing the lymphatic drainage of the right testicle (AP view) (dog injection). (**B**) Lateral view. Direct lymphangiogram of the testicle disclose the SLN in the precaval area (dog). (**C**) Bilateral direct testicular lymphangiogram in human concomitant with dorsum of the foot injection. The *arrows* indicate the location of the SLN in the *right* side as well as the *left*. (*see* Color Plate 16)

There is need for techniques to predict which patients are most likely have occult retroperitoneal disease. Positron emission tomography has been used in testicular cancer for the evaluation of post-operative beds to find residual tumor, but was found to be unreliable for staging *(46)*. The technique of lymphatic mapping with SN biopsy can potentially be the solution for the selection problem.

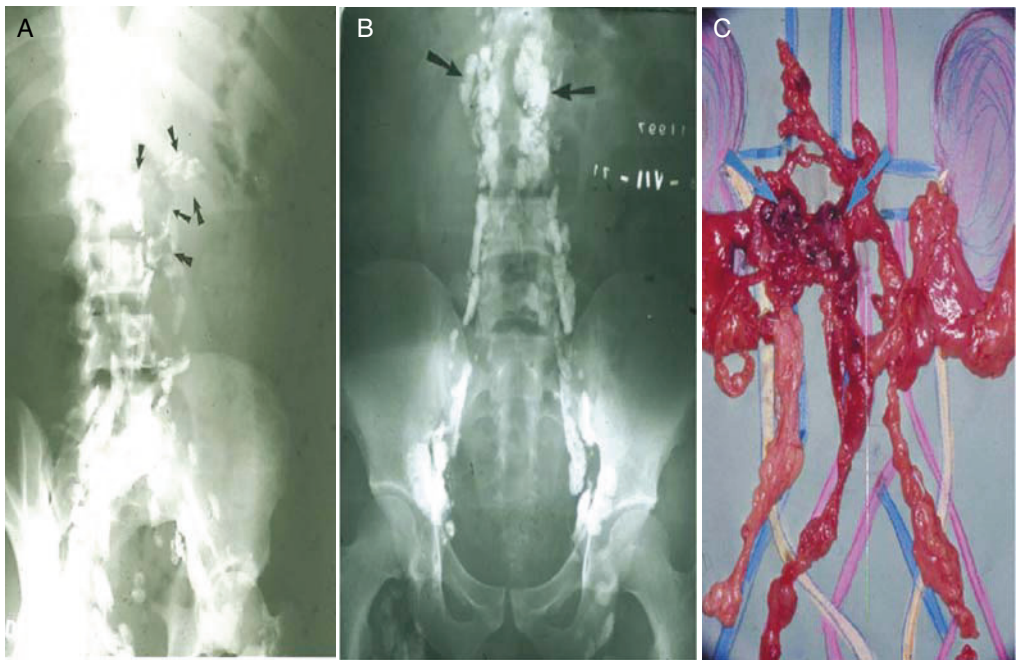

Fig. 3. (**A**) *Arrows* indicate SLN harboring metastasis (lateral view). (**B**) *Arrows* indicate SLN metastasis (AP view). (**C**) Surgical specimen of retroperitoneal lymph node dissection. *Arrows* indicate SLN. (*see* Color Plate 17)

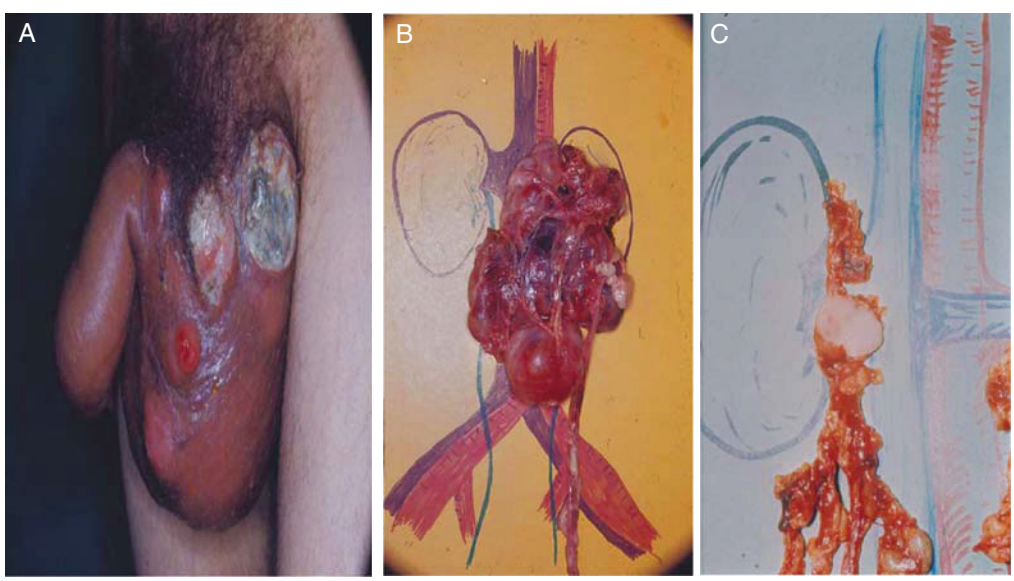

Fig. 4. (**A**) Advanced malignant neoplasia of the testicle (reluctant patient). (**B**) Metastasis lymph nodes pre-aorta–cava area. (**C**) SLN of the testicle harboring metastasis. (*see* Color Plate 18)

A single dose of ^{99m}Tc nanocolloid (Amersham Cygene, Eindhoven, the Netherlands) (or ^{99m}Tc-labeled phytate) in a mean volume of 0.22 ml (range 0.15–0.30) was injected with a fine needle into the funiculus in the first patient and into the testicular parenchyma in the following four patients *(56,64,75)*.

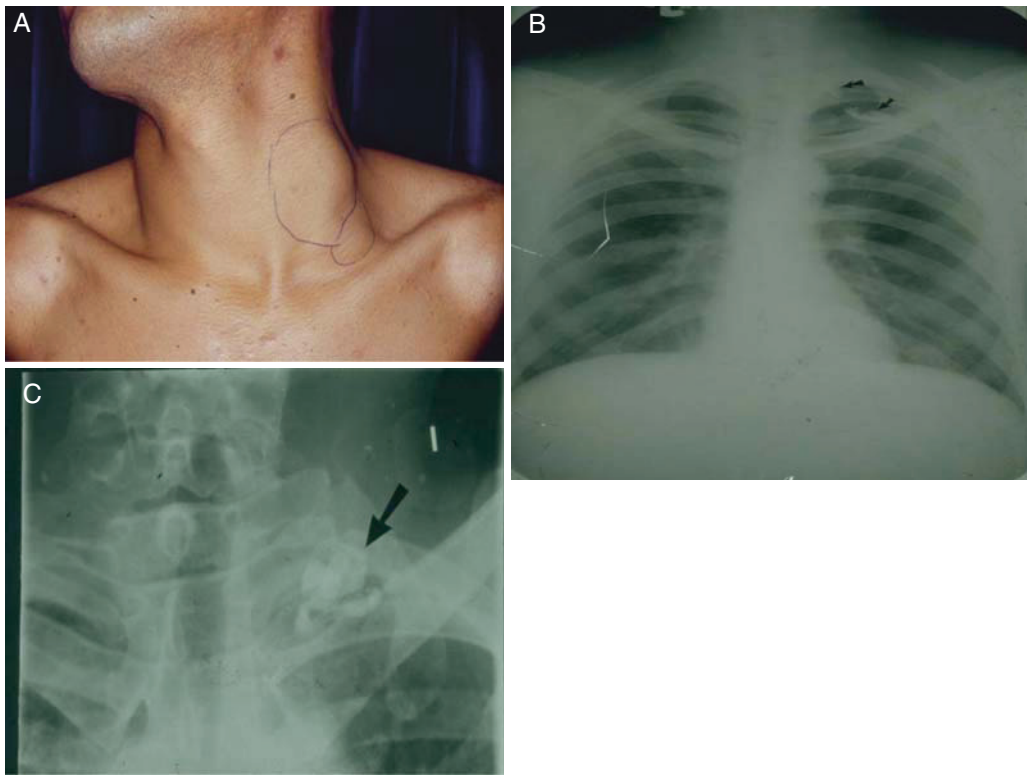

Fig. 5. (**A**) Palpable lymph nodes left supraclavicular area (photography). (**B**)< Chest X-ray. *Arrows* indicate supraclavicular SLN after testicular lymphangiogram. (**C**) Close detail of the supraclavicular SLN after testicular lymphatic injection.

Shortly after injection, anterior and lateral dynamic 20-s images were obtained with a dual-head gamma camera. Late static images were obtained 2–24 h after injection *(75)*.

Lymphoscintigraphy was followed by intraoperative lymphatic mapping via a transperitoneal laparoscopic approach on the same day. The SN was identified with the aid of intratesticular injected patent blue dye in a volume of 1.0 ml and an endoscopic gamma ray detection probe *(75)*.

Following the lymphoscintigraphy, a laparoscopic retroperitoneal SN biopsy in the same session as well as orchiectomy was carried out.

Preliminary data show that lymphoscintigraphy for SN identification is feasible in Stage I testicular cancer using intratesticular radiocolloid administration in combination with laparoscopic SN biopsy. Identification of occult LN metastases may prevent undertreatment or overtreatment in the future *(56,65,75)*.

The management of regional LNs in patients with clinical Stage I testicular carcinoma is a controversial problem. Several authors investigated the feasibility and accuracy of radioguided mapping of SLNs for men with clinical Stage I testicular tumors *(56,64,75)*.

Satoh et al. studied 22 patients with clinical Stage I testicular carcinoma *(64)*. A day before surgery ^{99m}Tc-labeled phytate was injected around the testicular tumor. After undergoing radical orchiectomy, patients underwent laparoscpic retroperitoneal LN dissection.

Nearly all SLNs were detected at the ventral or lateral side of the vena cava or at the aorta between the levels of L1, L2 to the aortic bifurcation. All SLNs were detected easily in a surgical procedure. Two patients had micrometastasis only in SLNs *(64)*.

Radioguided mapping of SLNs with laparoscopy was feasible, and nearly all SLNs were detected accurately by the procedure. In the near future, the standard retroperitoneal LN dissection may be avoided in most patients with clinical Stage I testicular carcinoma by utilizing focused examination of SLNs *(64)*.

6. RENAL CARCINOMA

Sherif et al. *(67)* showed special interest in the field of SLNB in renal cell carcinoma and he concluded that "SLN dissection in renal tumors is feasible, still evaluation of different modes of detection needs refinement and standardization."

7. CONCLUSION

The enthusiasm in investigating the biological phenomenon of the micrometastasis is overwhelming. Further analysis, and definitive conclusion have not been reached as yet, again the cooperation, understanding, and investigation between large research centers are necessary for the final and definitive result.

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31 Sentinel Lymph Node Mapping in Colorectal Cancer

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Contents

ABSTRACT

Sentinel lymph node (SLN) mapping has been widely applied in the staging of solid neoplasms including colon and rectal cancer. Since the first reported feasibility study in 1997, there have been numerous publications validating SLN mapping as a highly accurate and powerful upstaging technique for colon and rectal cancer. In addition to refining the technical aspects of this procedure, these studies have investigated the use of other tracers and operative techniques, while determining the indications, limitations, and pitfalls of SLN mapping in patients with colorectal cancers.

This chapter reviews the rationale for performing SLN mapping for the accurate staging of colon and rectal cancers, and provides a brief review of the historical background of the

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development of the procedure. We will focus on the technical details of the procedure, and on the pathological evaluation of the specimen and the SLNs. The various tracers and techniques of SLN mapping in colon and rectal cancer will be discussed. We have performed SLN mapping in more than 600 consecutive patients over the past 10 years. The success rates for identifying at least one SLN for colon and rectal cancer were 99.2 and 92%, respectively. The accuracy rates were 93.1 and 95.2%, respectively. In terms of upstaging, 24.2% of the colon cancer patients with nodal metastases and 25.9% of rectal patients with nodal metastases were upstaged by the detection of micrometastases found in the SLNs only.

Key Words: sentinel lymph node mapping; colorectal cancer; micrometastasis

1. INTRODUCTION

Colorectal cancer is the third most common cancer in men and women and the second most common cause of cancer death *(1)*, with an estimated 148,810 new cases and 49,960 deaths in the year 2008. The age-adjusted incidence rate is 50.6 per 100,000 men and women per year based on cases diagnosed in 2001–2005, with an age-adjusted death rate of 18.8 per 100,000 men and women per year. The overall 5-year survival rate is around 65% and about 6% of Americans are expected to develop the disease during their lifetimes *(2)*.

As with other solid cancers, the stage of the disease plays an important role in the prognosis with the lymph node status being the strongest prognostic indicator. The survival of node positive disease (AJCC [American Joint Commission on Cancer] III) decreases by 25–30% as compared to AJCC stage I and II disease (any T, N0, M0). The role of adjuvant chemotherapy has been shown to be curative in 30% of patients with stage III (any T, N1, M0) disease *(3,4)*.

Therefore, the diagnostic accuracy of nodal metastasis remains essential and critical for the proper prediction of survival as well as for appropriate therapeutic planning.

Although surgery alone should be considered curative in localized disease without nodal metastasis (AJCC stage I and II), about 10–25% of these patients will develop progression of their disease and will ultimately succumb to distant metastases within 5 years from the time of surgery. This group of patients is the basis of our assumption that many of these patients may have occult metastasis in the lymph nodes that is not detected by the conventional surgery without sentinel lymph node (SLN) mapping and by the conventional pathological examination of the lymph nodes. We hypothesize that a small volume metastasis (micrometastasis) in the lymph nodes or the SLNs is biologically significant and needs to be investigated more.

2. LYMPH NODE MICROMETASTASIS IN COLON CANCER

Since lymph node metastasis (>2 mm focus) is the strongest predictor of prognosis in colorectal cancer, it is reasonable to take a closer look at the prognostic significance of nodal metastasis measuring 0.2–2 mm, or what is labeled as "micrometastasis" by AJCC 6th edition criteria (Table 1).

Many researchers have used various pathological methods to enhance the detection rates of nodal micrometastases. These include serial sectioning *(5,6)*, immunohistochemistry (IHC) using cytokeratin *(7,8)*, and most recently, reverse transcriptase polymerase chain reaction (RT-PCR) *(9–11)*.

Despite these studies, the influence of lymphatic micrometastases on prognosis was not clearly established. While some studies have shown micrometastases can adversely affect outcomes, several other studies have been unable to show such a correlation. Most of these studies were limited by small sample size and/or lack of standard pathological techniques.

Table 1
Regional Lymph Node Pathological Staging System According to AJCC 6th Edition in Cancer:

pNX	Regional lymph nodes cannot be assessed
pN0	No regional lymph node metastasis histologically, no additional examination for isolated tumor cells
pN0(i–)	No regional lymph node metastasis histologically, negative IHC
pN0(i+)	No regional lymph node metastasis histologically, positive IHC, no IHC cluster greater than 0.2 mm
pN0(mol–)	No regional lymph node metastasis histologically, negative molecular findings (RT-PCR)
pN0(mol+)	No regional lymph node metastasis histologically, positive molecular findings (RT-PCR)
pN1	Metastasis in 1 to 3 axillary lymph nodes, and/or internal mammary nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent
pN1mi	Micrometastasis (greater than 0.2 mm, none greater than 2.0 mm)
pN1a	Metastasis in 1 to 3 axillary lymph nodes
pN1b	Metastasis in internal mammary nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent
pN1c	Metastasis in 1 to 3 axillary lymph nodes and in internal mammary lymph nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent
pN2	Metastasis in 4 to 9 axillary lymph nodes (pN2a) or in clinically apparent internal mammary lymph nodes in the absence of axillary lymph node metastasis (pN2b)
pN3	Metastasis in 10 or more axillary lymph nodes (pN3a), or in infraclavicular lymph nodes (pN3a), or clinically apparent ipsilateral internal mammary lymph nodes in the presence of 1 or more positive axillary lymph nodes (pN3b), or in more than 3 axillary lymph nodes with clinically negative microscopic metastasis in internal mammary lymph nodes (pN3b), or in ipsilateral supraclavicular lymph nodes (pN3c)

A meta-analysis was performed of all existing studies to better determine the prognostic value of micrometastatic lymphatic disease in patients with AJCC stage II colorectal cancer *(12)*.

All studies in the meta-analysis were able to identify micrometastases after subjecting pN0 lymph nodes to greater pathologic scrutiny. Molecular techniques using RT-PCR upstaged 37% of patients from pN0 to pN0(mol+) and were associated with an absolute survival difference at 3 years of 18.7%. Overall survival at 3 years was 78% for patients with molecularly detected micrometastases and 97% for patients without molecularly detected micrometastases ($p < 0.001$). Histologic techniques including serial sectioning with IHC staining upstaged 32% of node-negative (pN0) patients. Although micrometastases identified with IHC techniques appeared to adversely affect survival the differences were not statistically significant, possibly due to variations in IHC techniques. These variations included differences in the nodal counts per specimen, number of nodes analyzed with IHC per specimen, volume of nodal analysis, inadequate examination of individual lymph nodes, the range of anticytokeratin antibodies used, and the different definitions used to define micrometastasis. These results imply that lymphatic micrometastasis is a biologically important event and support the theory that micrometastases from colorectal cancer have prognostic significance.

These various techniques have indeed increased the rate of detection of nodal micrometastases in colorectal cancer, but with an enormous burden to the pathologist in terms of time, cost, and labor intensity. Therefore, it is impractical to apply such advanced techniques arbitrarily to just any or all lymph nodes within a specimen. It is reasonable, however, to apply these techniques on the SLNs as we are providing the pathologist with lymph tissue that is most likely to harbor metastases when present.

The identification of an adequate number of lymph nodes within a specimen by the pathologist also remains a major obstacle in the accurate staging of colon and rectal cancer. This problem is augmented by the fact that most histologically positive lymph nodes are less than 5 mm in size and are therefore difficult to identify *(8,13)*. While pathological methods such as fat clearance techniques *(14)* and pinning and stretching techniques *(15)* have been developed to increase the yield of lymph nodes per specimen, they also remain highly time and labor intensive. The SLN mapping technique therefore provides an ideal avenue for improving staging accuracy in patients with colorectal cancer.

3. SENTINEL LYMPH NODE MAPPING IN COLORECTAL CANCER

3.1. Historical Review

Although the term "sentinel node" was first used by Gould and colleagues *(16)*, the physiologic concept of SLN mapping was proposed and described by Cabanas *(17)* in 1977 for the treatment of penile cancer. In 1992, the definition of an SLN and the technique of lymphatic mapping were reestablished by the work of Morton and colleagues *(18)* in patients with malignant melanoma. Since the 1990s, this technique has been widely used for the accurate staging of nodal metastasis in a multitude of solid tumors including breast cancer *(19,20)*, colon and rectal cancers *(21)*, gynecological malignancies *(22,23)*, thyroid cancer *(23)*, prostate cancer *(24)*, lung cancer *(25)*, gastric and esophageal cancers *(26–28)*, pancreatic and small bowel cancers *(28)*, and anal canal cancer *(29)*.

By the late 1990s, evaluation of SLN mapping in melanoma, breast cancer, and colorectal cancer had been established through multiple studies, which investigated the rates of success, accuracy, sensitivity, specificity, and skip metastasis for the technique *(18,20,30,31)*. Since then, SLN mapping in colon and rectal cancer has been shown in multiple studies to be a powerful upstaging tool*(32–36)*.

The high accuracy of SLN mapping in melanoma and breast cancer helps avoid the morbidity associated with regional lymph node dissection SLN negative patients. All colorectal cancer patients undergo conventional lymph node dissection at the time of surgery.

However, since regional lymph nodes are not being dissected when the SLNs are negative in melanoma and breast cancer, the presence of false-negative SLNs "skip metastasis" will remain undiagnosed at the time of surgery and eventually present as recurrence in the regional lymph nodes. In colorectal cancer, all regional lymph nodes are dissected at the time of surgery and hence the false-negative sentinel nodes "skip metastasis" are detected at the time of surgery and therefore have no impact on the treatment.

The occurrence of aberrant drainage in colorectal tumors, for which the operation should be changed to extend the margins of resection, has been shown to be a very relevant yet infrequent occurrence *(37)*.

Studies were also conducted to evaluate the usefulness of various different tracers such as fluorescein, Lymphazurin, technetium sulfur colloid (TSC), and, recently, methylene blue as enhancers or alternates, and to explore new techniques such as laparoscopic, ex vivo, and minimally invasive approaches to SLN mapping for colon and rectal tumors.

3.2. Definition of a Sentinel Lymph Node

The SLN is defined as the first through fourth node(s) to receive direct drainage from the primary tumor site and which have the highest potential of harboring metastatic disease. Since it is impractical to extensively examine all of the lymph nodes by multilevel sections, IHC or RT-PCR, the SLNs can be meticulously examined by the pathologists with detailed analysis by multilevel sections, IHC, or RT-PCR methods. This may lead to the detection of occult nodal micrometastatic disease, which may have otherwise remained undetected by conventional pathological examination of a single section of the lymph node.

Detection of sentinel node metastasis or micrometastasis may upstage a significant number of patients with early stage colon and rectal cancers (from AJCC stage I/ II to stage III disease). These patients can then be offered potentially curative systemic chemotherapy, possibly leading to improved survival rates. Also, negative SLNs can more accurately define true stage I and II disease where no further chemotherapy is needed.

Recent changes in the AJCC 6th edition Cancer Staging Manual categorized and properly defined micrometastasis in breast cancer: pN1mi, pN0(i+), and pN0(mol+) (Table 1). An SLN/ was considered positive if it harbors cancer cells with a focus between 0.2 and 2.0 mm detected by hematoxylin and eosin (H&E), ultrastaging, IHC, or RT-PCR. The same definition of a positive SLN was used in melanoma *(38)*, breast cancer *(39,40)*, and colorectal cancer *(32,41)*.

Over the past 10 years, our group has undertaken a prospective study *(42,43)* regarding the usefulness of the SLN mapping technique for accurate staging of colon and rectal cancer. The objectives of our research are as follows:

- To determine the feasibility of the SLN mapping technique in colon and rectal cancer by utilizing isosulfan blue dye, or methylene blue.
- To assess the accuracy of the technique for determining the correct status of the regional lymph node basin.
- To identify any aberrant mesenteric lymphatic drainage patterns requiring any extension of the planned resection margins.
- To determine the indications, contraindications, limitations, and pitfalls of the technique.
- To identify the impact of SLN mapping on the recurrence of the disease.
- To evaluate the causes of skip metastasis "false-negative SLNs" and its relevance on the treatment.
- To compare the accuracy, nodal positivity, sensitivity, false-negative rate, and adverse reactions between different dyes used in SLN mapping.

3.3. Technique of Sentinel Lymph Node Mapping in Colon Cancer

From October 1996 through December 2006, 621 consecutive patients with the diagnosis of colorectal cancer were prospectively entered under an IRB approved protocol after informed consent was obtained (508 patients with colon cancer and 113 with rectal cancer). Preoperative evaluation for all patients included a complete history and physical examination, routine laboratory studies including liver function studies and carcinoembryonic antigen, colonoscopy, and computed tomography of the abdomen and pelvis. Prior to surgery, all patients were given standard bowel preparation along with prophylactic oral and intravenous antibiotics.

Intraoperative steps for the SLN mapping technique for colon cancer are the same irregardless of tumor location. The tumor site is identified at the time of laparotomy either by manual palpation of the tumor or by Preoperative endoscopic tattooing of patients who underwent polypectomy. The extent and size of the primary tumor as well as the presence of any distant metastasis are evaluated. The tumor-bearing portion of the colon is mobilized by dividing the

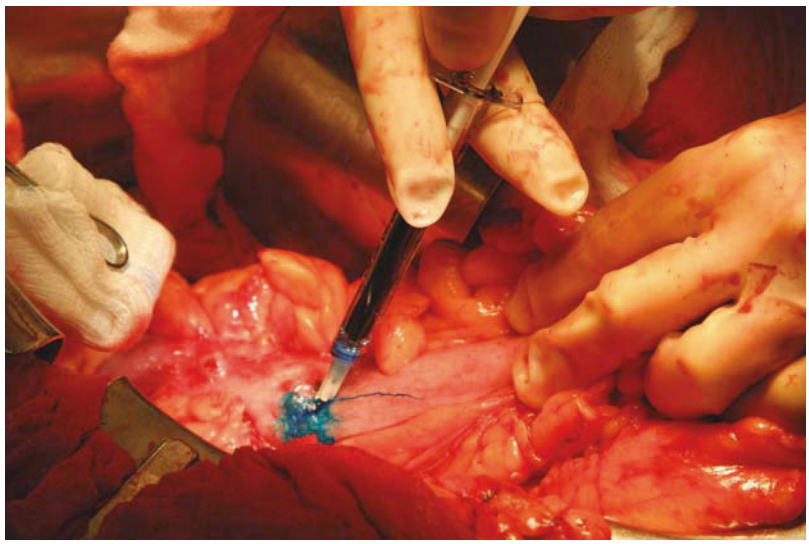

Fig. 1. Lymphazurin 1% is being injected in the subserosal layer of right colon. (*see* Color Plate 19)

lateral peritoneal attachments and any adhesions present. Utmost precautions are taken to avoid cutting through the peritoneum covering the mesentery in order to avoid any disruption of the mesenteric lymphatic pathways.

Once the tumor-bearing area of the colon is isolated, 1–4 ml of the dye (e.g., methylene blue 1% or Lymphazurin 1%) is injected using a tuberculin syringe and a 30-gauge needle (Fig. 1 and Color Plate 19). The dye is injected subserosally and circumferentially around the primary tumor. Special attention should be given when the tumor is at a mesenteric location, since circumferential injection of the dye is not always feasible. Great care is taken to avoid any spillage of the dye into the bowel lumen. This was studied by Joosten and colleagues in 1999 *(44)*, who found that intraluminal injection of the dye can lead to its absorption away from the primary tumor and may highlight lymph nodes that are not the "true" SLNs, leading to higher "skip metastases" rates and lower accuracy rates.

Within 5–15 min after the injection, the blue dye travels via the lymphatics to the nearby mesenteric lymph nodes, which stain pale to deep blue. The first to fourth blue staining lymph nodes are marked with sutures as "SLNs" (Fig. 2 and Color Plate 20). These are most often seen on the retroperitoneal surface.

It is important to tag SLNs with suture intraoperatively because SLNs lose their blue tinge rather quickly as the dye passes through the lymphatics and further downstream. Hence, the dye may be found in a lymph node down the lymphatic chain by the time the specimen reaches the pathologist, while the true "SLNs" may no longer be blue at all. This leads to failure of identification of the true SLNs by the pathologist. In the event that in vivo identification of one or more SLNs is not accomplished during the operation, an additional 1–2 ml of the blue dye (methylene blue or other dye) can be injected ex vivo. This might allow the pathologist to identify an SLN during pathologic dissection of the mesentery.

Once SLN mapping is completed and the SLNs are identified, a standard oncologic resection is performed including adequate proximal and distal margins of the bowel, along with resection of the regional lymph nodes in the attached mesentery. Occasionally, a blue node is identified outside of the usual lymphatic-bearing area and should also be considered as an SLN and

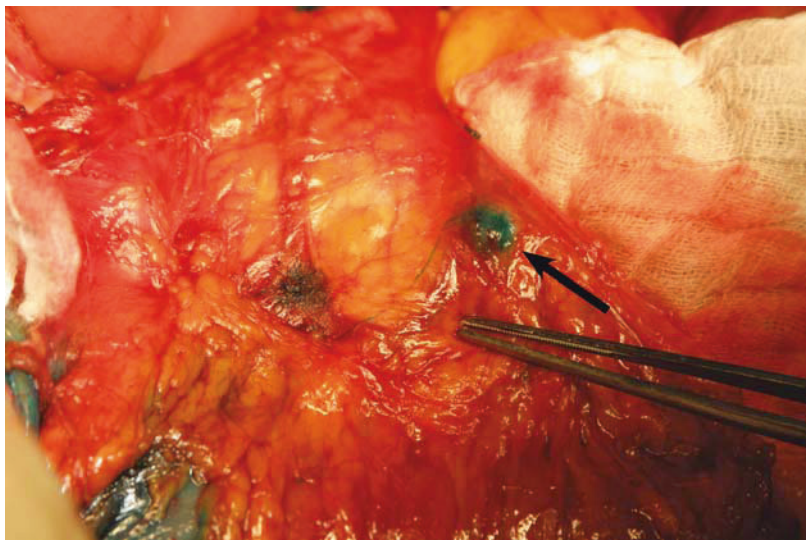

Fig. 2. Blue lymphatic traveling to a blue sentinel lymph node (*arrow*) seen on the retroperitoneal surface. (*see* Color Plate 20)

included within the margins of the resection. In patients with unusually thick or fatty mesentery, limited surgical dissection of the mesenteric fat may be required to identify the blue-stained lymph nodes.

3.4. Technique of Sentinel Lymph Node Mapping in Rectal Cancer

SLN mapping in rectal cancer is different according to the portion of the rectum involved by the tumor. For patients with tumors at the rectosigmoid junction or above the peritoneal reflection, SLN mapping is performed in a similar way as for colon cancer.

However, the technique is different for low to mid rectal tumors (located below the peritoneal reflection). The blue dye is injected with a 25-gauge spinal needle through a proctoscope into the submucosal and muscular layers of the tumor. If any blue nodes are visualized during mobilization of the rectum, they should be immediately marked with suture as SLNs. In many patients, especially those with low rectal tumors, no blue nodes outside of the mesorectum are usually found during total mesorectal excision (TME). In these cases, an is done first by TME, followed by the ex vivo submucosal injection of an additional 1–2 ml of the blue dye. Any blue nodes near the tumor within the mesorectum found ex vivo either by the surgeon or by the pathologist may be regarded as an SLN.

3.5. Dyes Used in Sentinel Lymph Node Mapping in Colorectal Cancer

Several tracers have been used for SLN mapping in colorectal cancer. These include Lymphazurin 1%, Fluorescein 10%, Patent blue, TSC, and Methylene blue 1%. Lymphazurin 1% has been the most frequently utilized dye for lymphatic mapping in colon and rectal cancer. The widespread use of the dye has led to rare reports of anaphylactic reactions *(45–47)* as well as interference with pulse oxymetry monitoring *(48,49)*.

Fluorescein 10%, on the other hand, has been used in some countries where Lymphazurin 1% is not easily available. Therefore, we tried to validate the use of Fluorescein 10% as an alternative to Lymphazurin 1% for SLN mapping in colorectal cancer. *(50)*. Fluorescein 10% is commonly available throughout the world, comparatively inexpensive, and is not associated with any

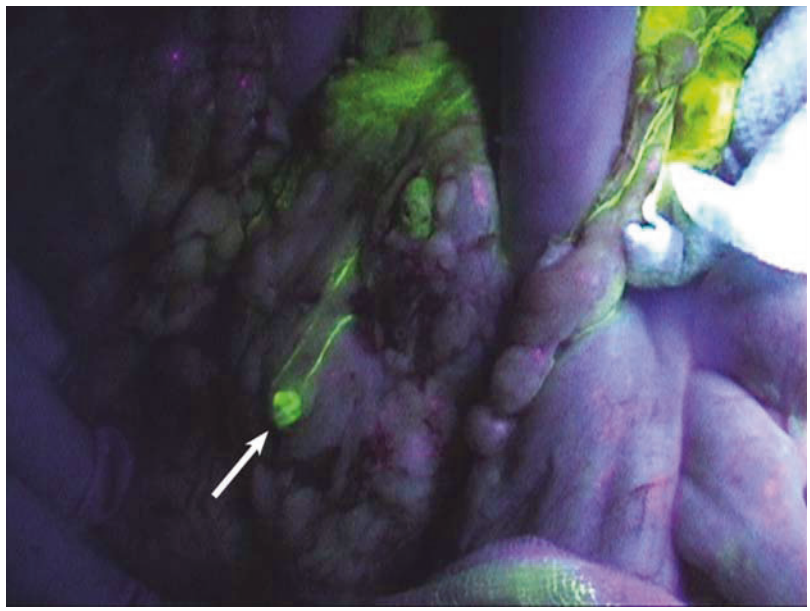

Fig. 3. Fluorescent lymphatic reaching a fluorescent sentinel lymph node (*arrow*) seen in a dark room with Wood's Light illumination. (*see* Color Plate 21)

known allergic reactions. We compared the use of fluorescein 10% in 120 of our patients to Lymphazurin 1%. The technique of injection of both dyes is similar using a tuberculin syringe and 1–4 ml of the dye. The dye travels quickly via the lymphatics and turns the SLNs fluorescent, which can be visually identified in a dark room under Wood's light illumination as bright yellow nodes (Fig. 3 and Color Plate 21). Our results were comparable between Lymphazurin and fluorescein. No allergic reaction has been observed during the use of either Lymphazurin or Fluorescein dye *(50)*.

As TSC has been shown to be used in SLN mapping for melanoma *(51)* and breast cancer *(19)*, it can also be used for lymphatic mapping in colorectal cancer. Radioactive dye has been evaluated in SLN mapping for colorectal cancer in few studies *(52,53)*. We have studied the efficacy of TSC in 57 of our patients. 0.5–1 mCi of TSC was injected using a guarded syringe in a similar way as with Lymphazurin and Fluorescein *(54)*. Identification of an SLN was based on increased radioactivity "hot nodes" detected with the use of a gamma probe. Kitagawa et al. *(52)* in Japan has also used technetium tin colloid for successful lymphatic mapping in colon cancer. We have found that TSC is a useful adjunct to Lymphazurin. Although the identification of many "hot" nodes is facilitated by their blue coloration when Lymphazurin is also used, TSC radioactive nodes have a comparable accuracy in correctly predicting the status of the nodal basin. As a matter of fact, we found that those SLNs that are both "blue" and "hot" are about twice as likely to yield histologic positivity, and may represent SLNs that should receive particularly special attention from the pathologist.

Since Methylene blue 1% has been shown to be effective in SLN mapping in melanoma and breast cancer, and because of the recent unavailability of Lymphazurin 1%, we conducted a comparative study of these dyes. Our study aimed to evaluate the efficacy of Methylene blue 1% in SLN mapping in colorectal cancer and compared it to that of Lymphazurin 1%. Methylene blue 1% was injected in a similar way to Lymphazurin 1% in the last 60 patients, and the results of SLN mapping were comparable between the two dyes for colorectal cancer. The most common adverse reaction with Lymphazurin 1% was pseudohypoxemia. Though local skin

necrosis is a common side effect with Methylene Blue 1% for breast cancer, there was no such adverse reaction in colorectal cancer. There were no anaphylactic reactions associated with either dyes *(55)*.

3.6. Ex Vivo Mapping

Ex vivo mapping was originally described by Wong et al. *(56)* as an alternative to in vivo mapping. After a standard oncologic resection, the specimen is dissected by the pathologist along the antimesenteric border, regardless of whether the tumor is along that line. In their study, Wong and colleagues injected Lymphazurin 1% at four quadrants submucosally, with 0.25 ml of dye injected at each location. The specimen is then gently massaged for 5 min. All identified blue nodes are then designated as SLNs. The success rate to identify at least one SLN was 92.3% (24/26), with an average of 3.0 SLNs per patient. Nodal metastasis was not detected in 14 patients in their study and therefore, advanced pathological methods (ultrastaging and IHC) were employed in these patients. Of the 14 patients, 29% (4/14) were upstaged by the identification of nodal micrometastases. Of these, 14% (2/14) were only identified by IHC means.

3.7. Laparoscopic Experience

Lymphatic mapping has also been described in colon tumors by some authors using laparoscopic techniques *(57,58)*. The dye may be injected into the submucosa by endoscopy or into the subserosal layer under laparoscopic visualization. Kitagawa et al. *(26,59)* have described the technique for successfully locating the SLNs using a laparoscopic gamma probe while others have used Lymphazurin 1%. Wood and colleagues reported a success rate of 100% as well as an accuracy rate of 100% in a series of nine patients who underwent laparoscopic SLN mapping with an average of two SLNs identified per patient. As laparoscopic colectomy for early colon cancer becomes more common, the importance of these techniques will increase, especially in cases where the root of the mesentery is difficult to resect laparoscopically (i.e., morbid obesity).

3.8. Pathological Examination of SLNs

From the pathologist's perspective, the principal goal of evaluating colorectal cancer specimens is to provide accurate data regarding the staging and prognosis of the disease This includes details regarding the tumor and its extent, appropriate margins, and regional lymph node dissection.

Accurate assessment of the regional lymph nodes depends on the number of lymph nodes harvested since predictive probability of detecting lymph nodes involved by the tumor depends greatly on the number of nodes harvested at the time of surgery *(60)*. There is no absolute minimum number of nodes obtained that can guarantee identification of all nodal metastasis. Therefore, pathologists have become more aware of using various methods to increase the sensitivity of detecting nodal metastasis, such as stretching and pining of the mesentery for fixation, or using fat-clearing agents (i.e., Carnoy's fluid) to render the nodes visible within the mesenteric fat. However, both of these methods require extra time, effort, and cost.

The pathologist's attention can, however, be directed to one to four SLNs that will most likely harbor metastasis by using SLN mapping in colorectal cancer. Applying advanced pathological techniques such as ultrastaging, IHC, or RT-PCR on the SLNs, along with routine dissection of the remaining regional lymph nodes, improves the detection of nodal metastasis, and thus improves staging accuracy.

Upon receipt of the surgical specimens into our pathology department, the SLNs were dissected free from the specimen, sectioned grossly at 2- to 3-mm intervals and blocked separately

in individual cassettes. The remainder of the specimen was evaluated in sandard fashion. Harvested non SLNs were dissected and formalin fixed according to standard pathologic protocols. For some cases, postfixation of the pericolic adipose tissues for 2–18 h in Carnoy's fluid aided in the identification and retrieval of non-SLNs, which appeared white against the pale yellow background of fat *(61)*.

Five sections were prepared at 20- to 40-μm intervals for each of the SLNs, the first 4 were stained routinely with H&E, while the last section was labeled for cytokeratins by IHC (AE-1/AE-3 cocktail; Ventana Medical Systems, Tucson, AZ). Other sections of the tumor and non-SLNs were routinely stained with H&E. For some studies, frozen sections of representative SLNs, non-SLNs, and tumor were obtained for RT-PCR analysis.

3.9. The Swiss Trial: The Amount of Dye is Related to the Size of the Tumor

The true benefit of SLN mapping in colorectal cancer lies in its ability to identify the SLNs with micrometastatic disease, which would remain undetected if the nodes were examined by conventional techniques. Hence, such nodal micrometastases are more likely to be detected in early T1, T2, or T3 tumors, rather than in more bulky T4 lesions or tumors invading adjacent structures. In such large tumors, however, the amount of dye injected circumferentially would be higher than the usual 2 ml. A Swiss group *(62)* first tested the adequate amount of dye needed in relation to the size of the tumor.

Two factors were tested in this trial for lymphatic mapping in colorectal cancer: the amount of the injected dye in relation to the diameter of the primary tumor and the relationship of the SLN status with bone marrow micrometastases. In this trial *(62)*, at least 0.5 ml of the blue dye per one centimeter of tumor diameter was found to be most successful in identifying at least one SLN. Hence, some of the higher failure rates seen in some published series may indeed be due to the inadequate amount of dye injected relative to the size of the tumor. We agree that at least 0.5 ml of dye for each cm of tumor diameter is reasonable for accurate lymphatic mapping, especially for larger tumors. The addition of radiocolloid did not increase the success rate of SLN mapping in identifying the true SLNs.

Although bone marrow micrometastasis has been found to be of prognostic significance in breast cancer, such a correlation was lacking for colorectal cancer. In this trial, a higher incidence of positive bone marrow micrometastases was detected in patients with positive SLNs than in negative SLNs (50 vs. 27%), with no statistical significance however.

3.10. Skip Metastasis

Skip metastasis or false-negative rates is defined as the failure to identify cancer cells in the SLNs when they are subsequently identified in the non-SLNs. For statistical purposes, skip metastasis is calculated as (false negative/[false negative + true positive]).

Unlike in melanoma and breast cancer, regional lymph nodes are resected at the time of operation in colorectal cancer, regardless of the SLN status. Therefore, it is not possible to calculate the rates of skip metastasis at the time of surgery and the presumptive rate of skip metastasis in melanoma and breast cancer equals the rate of regional lymph nodes recurrence in patients with initial negative SLNs.

Skip metastasis rates have been reported to be between 7.8 and 10% in multiple studies *(40,63,64)* in breast cancer and also in melanoma *(38)* where these patients presented with regional recurrence. Skip metastasis in colorectal cancer has been evaluated in different studies *(35,65–68)* Most of these studies reported skip metastasis rates between 10 and 25%.

In breast cancer and melanoma, patients with skip metastasis remain understaged and undiagnosed as the regional lymph nodes are not dissected when the SLNs are negative at the

time of the initial operation. These patients may not receive adjuvant chemotherapy because of this understaging. Patients with colorectal cancer, however, undergo a standard oncological resection including regional lymph nodes dissection in addition to SLN mapping, and therefore all lymph nodes (SLNs and non-SLNs) are examined by the pathologist. Hence, even if skip metastasis is present, no patients will be pathologically understaged or undertreated. The biological significance of skip metastasis for colorectal cancer is minimal as compared to its high clinical relevance in breast cancer and melanoma.

4. MULTICENTER TRIALS EVALUATING SENTINEL LYMPH NODE MAPPING IN COLORECTAL CANCER

After multiple single-institution retrospective studies supported the hypothesis that the sentinel node theory could be successfully applied to colorectal cancer, multicenter SLN trials were created. Few major multicenter trials highlight the potential impact and limitations of SLN mapping in colorectal cancer. The Cancer and Leukemia Group B (CALGB 8001) performed a study where 25 surgeons at 13 participating centers enrolled 72 patients *(67)* .Although all surgeons had experience with SLN mapping, the majority performed fewer than five mapping procedures for colorectal cancer; only two surgeons performed more than ten mapping procedures. The rate of SLN identification was only 66% and the rate of false-negative mapping results was 54%. This study highlights the importance of documenting proficiency of individual surgeons not only in the technique and theory of SLN mapping but also, specifically, the practice of SLN mapping in colon and rectum.

Lim and colleagues *(69)* conducted a prospective cohort evaluation of SLN mapping in colon cancer between September 1998 and April 2006. They included 120 eligible patients, and a minimum of one SLN was identified in 119 patients leading to a success rate of 99%. The median number of SLNs identified per patient was four. Forty-nine patients had node-positive disease (SLNs and non-SLNs), of these only 29/49 patients had positive SLNs leading to a sensitivity of 59% while 20/49 patients had negative SLNs leading to a skip metastasis of 41%. The above analysis was based on H&E sections only and excluded results obtained by the ultrastaging of the SLNs. Upon evaluation of additional thin H&E sections of the SLNs, three more patients had SLNs metastasis. One of these three patients had an initial negative SLN.

Sixteen more patients had SLNs that were negative for metastasis on H&E staining but were positive on further IHC staining. Of these 16, eight had only IHC-positive SLNs (both SLNs and non-SLNs were negative for metastasis on routine H&E stain). After including patients with micrometastasis on the basis of thin H&E sections or IHC, the sensitivity would be 82% and the skip metastasis rate would be 18%. Stojadinovic and colleagues *(66)* conducted a randomized prospective study, between August 2002 and April 2006, comparing SLN mapping in colon cancer with standard pathologic evaluation without SLN mapping for the staging of colon cancer. The study included a total of 161 patients randomized to either arm.

Ninety-three patients were randomized to the SLN mapping arm. Nine patients were excluded, and 82 of the remaining 84 patients (97.6%) had successful SLN mapping with a median of two SLNs identified per patient. Out of these 82 patients, SLN metastasis was identified in 26 patients by conventional H&E section and in 47 patients by IHC and/or conventional H&E sections, therefore upstaging 57.3% of patients as compared to 38.7% of patients upstaged when conventional pathological methods with no SLN mapping was used, $p = 0.019$.

Bilchik et al. *(70)* reported preliminary results of a prospective multicenter trial (R01-CA90484) in 2006; four surgeons at three member institutions enrolled 132 patients. This study selected experienced surgical and pathological teams that had performed SLN mapping in colorectal cancer at least 20 times to participate. The experience of the surgeons and teams were reflected in the results. The sensitivity of lymphatic mapping was 88.2% and the false-negative rate was 7.4%. Micrometastases (i.e., pN1mi(sn) or pN0(i–)(sn)) were identified in 23.6% of patients whose nodes stained negative by H&E staining (pN0) and were considered upstaged.

5. OUR MULTICENTER TRIAL RESULTS

Our trial included 621 consecutive patients in four centers in which each surgeon had performed at least 30 cases each. In this study, 508 patients had colon cancer and 113 patients had rectal cancer. Their experience was reflected in the results. SLN mapping successfully identified one to four SLNs in 99.2% of colon cancer patients and 92% of rectal cancer patients. Of these 621 patients, one SLN was identified in 197 (32%) patients, two SLNs in 186 (30%), three SLNs in 131 patients (21%), and four SLNs in 69 patients (11%). In 25 patients (4%), more than four SLNs were identified.

6. COLON CANCER

Of the 508 patients with colon cancer, primary tumors were distributed as follows: appendix, 1; cecum, 119; right colon, 165; hepatic flexure, 7; transverse colon, 50; left colon, 30, and sigmoid colon, 136. Ages ranged from 40 to 97 years (median 73 years). The SLNs mapping technique successfully identified —one to four SLNs in 504 out of 508 patients (99.2%). A total of 7,772 lymph nodes were examined (mean: 15.3 nodes per patient), of which 1,118 (14.4%) lymph nodes were identified as SLNs.

Node-positive disease was found in 229 out of 433 patients with invasive cancer (71 patients with Tis/T0 tumor and 4 patients with SLN failure were excluded) leading to an overall nodal positivity of 52.9% (229 patients had node-positive disease out of 433 patient with invasive cancer). Out of these 229 patients with node-positive disease, SLN metastasis was found in 194 patients (true positive nodes). Eighty three out of these 194 patients (42.8%) had SLNs as the exclusive site of metastasis with all other non-SLNs being negative. In 47/194(24.2%) patients with histologically positive nodes, micrometastasis were identified by ultrastaging or IHC (micrometastasis is defined by nodal disease between 0.2 and 2.0 mm in diameter) (Table 2).

Out of the 508 patients with colon cancer, SLNs were negative for metastasis in 310 (61%) patients. Of these 310 patients, 275 had SLNs, as well as all the non-SLNs negative for metastasis leading to a negative predictive value of (87.1%). In the other 35 patients, the SLNs were negative but the non-SLNs were positive for metastasis leading to a skip metastasis rate of 15.3% (35/229 patients with node-positive disease).

The extent of surgery was altered by evidence of an aberrant lymphatic drainage detected by the SLN mapping technique in four colon cancer patients. Overall, the sensitivity of SLN mapping for colon cancer in our series was 84.7% (194 patients with true positive lymph nodes/229 patients with node-positive disease). The specificity was 100% and the negative predictive value was 87.1%. The accuracy of correctly predicting the status of the nodal basin was 93.1%. Micrometastasis in the SLNs was found in 24.2% of the 194 patients with histologically positive node, and thus may have upstaged these patients from AJCC stage I/II to stage III, allowing them to possibly benefit from adjuvant chemotherapy.

Table 2
Sentinel Lymph Node Mapping in Patients with Colon Versus Rectal Cancer

	Colon cancer	*Rectal cancer*	*Total*
Total no. of patients	*508*	*113*	*621*
Patients with successful SLNM	99.2% (504/508)	92% (104/113)	97.9% (608/621)
Patients with failure	0.8% (4/508)	8% (9/113)	2.1% (13/621)
Patients with skip metastases (false negatives)	15.3% (35/229)	15.6% (5/32)	15.3% (40/261)
Accuracy rate	93.1% (469/504)	95.2% (99/104)	93.4% (568/608)
Sensitivity	84.7% (194/229)	84.4% (27/32)	84.7% (221/261)
Specificity	100%	100%	100%
Negative predictive value	88.7% (275/310)	93.5% (72/77)	89.7% (347/387)
Patients with SLN as the exclusive site of metastases	42.8% (83/194)	48.1% (13/27)	43.4% (96/221)
Nodal positivity	52.9% (229/433)	33% (32/97)	49.2% (261/530)
Patients upstaged (micrometastases)*	24.2% (47/194)	25.9% (7/27)	24.4% (54/221)

SLNM = sentinel lymph node mapping.
*Patients with micrometastasis discovered by pathological ultrastaging of SLNs

7. RECTAL CANCER

Of the 621 consecutive patients, 113 had rectal lesions. Of these, 34 were in the rectosigmoid colon and 79 were in the rectum. Ages for this group ranged from 32 to 85 years (median 71 years). The SLN mapping technique successfully identified one to four SLNs in 104/113 patients (92%). In nine patients, the SLN mapping technique failed to identify any blue node (seven of these patients were treated with neoadjuvant chemoradiation therapy). The following analysis is based on the remaining 104 patients with rectal cancer in whom at least one SLN was identified. A total of 1,290 lymph nodes were examined (mean 12.4 nodes per patient), of which 229 (17.8%) lymph nodes were designated as SLNs. Out of the 104 patients with successful SLN mapping, seven were excluded since they had benign pathology.

Out of the remaining 97 patients, the overall nodal positivity was 33% (32 patients out of 97). In 27 patients the SLNs were positive for metastasis (true positive). In 13 out of the 27 (48.1%) patients with true positive SLNs, SLNs were the exclusive site of metastasis and in seven patients (25.9%), micrometastasis was identified by ultrastaging or IHC.

In 77 (74%) patients the SLNs were negative for metastasis. In 72 out of these 77 patients, the non-SLNs as well as the SLNs were negative for metastasis leading to a negative predictive value of 93.5%, while in five patients, the SLNs were negative and the non-SLNs were positive for metastasis leading to a skip metastasis rate of 15.6% (5 out of 32 patients with node-positive disease).

The extent of surgery was altered by evidence of an aberrant lymphatic pathway detected by the SLN mapping technique in three patients. Overall, the sensitivity, specificity, negative predictive value, and accuracy of SLN mapping for rectal cancer in our series were 84.4, 100, and 93.5%, respectively. Micrometastasis in the SLNs was found in 25.9% of the 27 patients with true positive sentinel nodal metastases, and thus may have upstaged these patients from AJCC stage I/II to stage III, allowing them to benefit from adjuvant chemotherapy (Table 2).

Out of the 40 patients with skip metastasis, 85% had T3 or T4 lesions. The average size of the tumor was 4.0 cm, and the right side of colon was involved by the tumor in 40% of cases. In 45% of patients, skip metastasis occurred in lymph nodes completely replaced by the cancer cells.

We compared our 621 patients who underwent SLN mapping (group A) to 517 patients who underwent conventional surgery without SLN mapping. (group B).

There were 508 colon cancer patients in group A as compared to 408 patients with colon cancer in group B. For patients with invasive disease (T1–T4), the total number of lymph nodes per patient was 12.4 in group B as compared with15.3 lymph nodes per patient in group A ($p < 0.0001$). The comparative analysis of nodal positivity based on T stage between the two groups in colon cancer is shown in Table 3.

There was a statistically significantly higher overall nodal positivity in patients with colon cancer in group A (52.9%) as compared with 37.5% for patients in group B ($p < 0.0001$) (Table 3).

Out of the 517 patients in group B, there were 109 patients with rectal cancer, as compared to 113 patients in group A. The average lymph nodes per patient were 12.4 in group A and 9.4 in group B. Table 4 compares the nodal positivity between both groups according to the T staging in rectal cancer. There was no statistical difference of nodal positivity between group A and group B for patients with rectal cancer, even though 81% of patients in group A underwent neoadjuvant chemoradiation therapy as compared to 40% of patients in group B (Table 4).

Table 3
Comparative Analysis of Nodal Positivity in Colon Cancer According to the T Staging: SLNM Versus Conventional Surgery

	SLNM (Gp A)		*Conventional (Gp B)*		
T stage	*No. of patients*	*% node + ve*	*No. of patients*	*% node + ve*	
Tis/SLNM failure	75	0	0	0	
T1	47	5 (10.6%)	57	4 (7%)	
T2	82	26 (31.7%)	61	9 (4.8%)	
T3	276	178(84.5%)	244	116 (47.5%)	
T4	28	20 (71.4%)	46	24 (52.2 %)	
Total (T1–T4)	433	229 (52.9%)	408	153 (37.5%)	$p < 0.001$

SLNM = sentinel lymph node mapping. (Full Size Table)

Table 4
Nodal Positivity in Rectal Cancer as Compared to the T-Staging: SLNM Versus Conventional Surgery

	SLNM (group A)		*Conventional (group B)*		
T staging	*Number of patients*	*Percentage of positive nodes*	*Number of patients*	*Percentage of positive nodes*	*p value*
Tis/SLNM failure	16	0	0	0	
T1	21	3 (14.3%)	12	2 (16.7%)	
T2	20	3 (15%)	25	5 (20%)	
T3	46	23 (50%)	59	26 (44.1%)	
T4	10	3 (30%)	13	7 (53.8%)	
Total (T1–T4)	97	32 (33%)	109	40 (36.7%)	0.66

Note: 81% of patients in the SLNM group received neoadjuvant chemotherapy as compared to 40% in the conventional group.

SLNM = sentinel lymph node mapping.

To evaluate the effect of multilevel sectioning of the SLNs only as opposed to the non-SLNs, 200 patients were enrolled in a study where the SLNs as well as the non-SLNs were examined using H&E stains, multilevel sectioning, and IHC in an identical manner with the pathologist being blinded to the original staging of the patients.

A total of 2,755 lymph nodes were examined. Of these, 494 (17.9%) were SLNs. The nodal positivity of the SLNs was 20.9% (103/494) as compared to 8.7% (196/2,261) for non-SLNs ($p < 0.001$). After ultrastaging all of the initially negative non-SLNs (2,065) in a similar way to the SLNs, only 0.6% (12/2,065) of them were found to have nodal micrometastasis. The remaining 99.4% remained histologically negative. Out of the 12 patients with micrometastasis, ten had previously detected nodal metastatic disease. As a result of that, the AJCC staging system was changed in only (1%) 2/200 patients secondary to the ultrastaging of non-SLNs. These results further confirm the unique distribution of metastasis via the lymphatics to the SLNs with minimal chance of skip metastasis.

8. CONCLUSION

Nodal metastasis is the strongest prognostic factor in colorectal cancer. Adjuvant chemotherapy is considered the standard of care for patients with nodal metastases in colorectal cancer and has been shown to reduce cancer-related mortality by about one third *(3,4)*. Hence, identification of such patients is the key for proper staging patients with colorectal cancer.

Technological and pathological advances over the past decade have made it possible to upstage patients from AJCC stages I and II to AJCC stage III by identifying nodal micrometastases using various techniques, such as, serial sectioning, IHC, and RT-PCR. The introduction of SLN mapping in colorectal cancer has made possible for the pathologist to focus their attention on the few nodes with the highest probability of harboring metastatic disease. Application of these techniques to all of the resected nodes within a specimen would be highly inefficient and costly.

SLN mapping in colorectal cancer can be performed with a success rate and accuracy rate of more than 90% if performed by an experienced surgeon. It significantly increases the nodal positivity and upstages a significant number of patients from AJCC I and II to AJCC III.

The failure of the technique to identify an SLN is often a result of preoperative chemoradiation therapy for mid- to low-rectal cancers and may be due to fibrosis of the submucosal lymphatics. Even if the skip metastasis rates in colon cancer are higher than breast cancer and melanoma, it has no clinical impact on the treatment since no patients will be understaged or undertreated. Most of the cases of skip metastasis occur in patients with T3 and T4 lesions, and often in advanced cases some lymph nodes may be completely replaced by tumor; hence, the dye may not penetrate those nodes. However, the blue lymphatics may lead the surgeon to those nodes and they should still be marked as SLNs. If the clinically positive nodes are detectable at laparotomy, then the mapping is not of any real benefit, as metastatic nodal disease is already evident. The relative limitations and contraindications of the procedures are listed in Table 5.

Table 5
Limitations and Contraindications of SLN Mapping

Limitations	*Contraindications*
• Previous colon surgery • Neoadjuvant chemo/radiation therapy • Large tumor invading adjacent organs • Perforated carcinoma • Multiple primary tumors	• Distant metastasis • Clinically positive lymph nodes

Unlike melanoma and breast cancer where the primary purpose of SLN mapping is to change the procedure, SLN in colorectal cancer is focused mainly to upstage patients and precisely identifies patients with truly node negative disease. As the technical aspects of the mapping are relatively simple, the overall learning curve of the SLN mapping technique in colorectal cancer is shorter. Adhering to the details of the intraoperative technique and the pathological evaluation are critical to decrease the incidence of skip metastasis. The fine-tuning of the technique over the past few years has included studies with alternative tracers and studies to determine the appropriate amounts of dye in relation to the size of the mass. The recent unavailability of 1% Lymphazurin, its associated risk of allergic reactions or interference with pulse oximetry, and the substantially higher cost as compared to 1% Methylene blue ($210 per patient in Lymphazurin vs. $7 per patient in methylene blue), make the latter a better choice in SLN mapping *(55)*.

TSC has also been successfully used for SLN mapping and has been found to be a helpful adjunct to Lymphazurin. Those nodes that are identified by both Lymphazurin and TSC have been shown to have a much higher yield for metastatic disease and deserve the special attention of the pathologist *(54)*.

Plans are underway for a large, multinational study from major centers in Europe, Asia, and USA to evaluate and to verify the efficacy of this technique and to assess its impact on the survival of patients with colorectal cancer. If verified in such trials, the application of the SLN mapping technique in colorectal cancer may become part of the standard practice of general surgeons given its simplicity, high accuracy, low cost, and especially its ability to aid the pathologists to focus their attention on one to four SLNs for detailed analysis and accurate prediction of nodal metastasis. Upstaged patients can be offered adjuvant chemotherapy, which may increase their survival.

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32 Application of Cancer Biology in Cancer Staging and Predicting Clinical Outcome

Frederick L. Greene, MD

CONTENTS

ABSTRACT

The importance of staging cannot be overemphasized in the management of patients. A primary role for staging is to stratify patients into groups that are prognostically and therapeutically similar. Without having this framework it would be difficult to have any meaningful clinical trials. A second goal for staging is that it allows for comparison across large populations either within geopolitical borders or between disparate countries. Tumor–node–metastasis (TNM) elements and the combination of these elements (stage group) serve as one of the most important prognostic factors in making assumptions relative to the overall survival of the cancer patient. The strategy is to apply well-tested evidence-based methodology to each factor to assess the significance and statistical power prior to applying the specific factor to the TNM lexicon. The traditional prognostic parameters for human breast cancer have stood the test of time and currently include specific tumor factors of size, lymph node status, and grade of tumor. The role of axillary nodal and lymphovascular involvement cannot be overemphasized in the prognosis and staging of many solid tumors. Current strategies have included the number of tumor-containing nodes in the locoregional area as well as the volume of tumor contained in these nodal elements. The introduction of artificial intelligence and the unification of concepts within the structure of nomograms will no doubt help to refine the language of cancer and will give a greater acuity of information to physicians, patients, and their caregivers.

Key Words: Staging; TNM; prognostic factors

> Disease is very old and nothing about it has changed. It is we who change, as we learn what was formally imperceptible – Charcot

The basic tenet of cancer staging emanated from the 1940s to the 1950s through the effort of a surgeon, Pierre Denoix, working in Paris *(1)*. This classification, based on characteristics of the tumor (T), nodal involvement (N), and metastatic findings (M), is an anatomical staging system

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that has been utilized for a variety of solid tumors and has now been promulgated worldwide since 1987 *(2)* through the work of the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC). Pediatric tumors are not included in this staging strategy. Tumors of the gynecological system although clinically staged by the classification of the International Federation of Gynecology and Obstetrics (FIGO) are included in the staging strategy. Currently, there have been six editions of both the UICC and AJCC publications and plans are currently being made for a seventh edition, which will be published in the spring of 2009 that will be utilized for patients diagnosed after January 1, 2010. The development of these staging strategies depends on new data which have been derived from large data sets and from the peer-reviewed literature. Groups of experts have been assigned to each specific tumor site and have made decisions relative to the inclusion of new anatomical markers and molecular or serum markers which have been introduced into the management strategies for a variety of tumors *(3)*.

The importance of staging cannot be overemphasized in the management of patients. A primary role for staging is to stratify patients into groups that are prognostically and therapeutically similar. Without having this framework, it would be difficult to have any meaningful clinical trials. A second goal for staging is that it allows for comparison across large populations either within geopolitical borders or between disparate countries. In view of the fact that the current anatomical (TNM) staging strategy is worldwide, this opportunity for comparison becomes even more important. Thirdly, the staging allows a framework for discussion especially among physicians who care for the individual patient. Staging in fact is our "language of cancer" and it is necessary for that language to be learned early in one's medical education in order to allow clinicians to become facile with the dialect and vernacular of staging strategies.

One of the criticisms of the current TNM system is that it may be biologically too simple. We know that generally a tumor will grow locally and expand in a locoregional manner. Following this local growth there may or may not be orderly involvement of regional lymph nodes although the mechanisms of this spread are yet to be fully elucidated. Finally, tumors spread to visceral organs and may or may not involve surrounding lymphovascular structures. The biology of most tumors is certainly more complex than this simple strategy although the basis of the TNM system has stood the test of time. Basic TNM elements and the combination of these elements (stage group) serve as one of the most important prognostic factors in making assumptions relative to the overall survival of the cancer patient. One definition of a prognostic factor is that it "serves as a variable that can explain some of the heterogeneity associated with the expected course and outcome of a disease" *(4)*. This factor has a role in foretelling the future of the specific cancer patient, but must be modified and modulated by other important biological factors which are currently being assessed.

One of the difficulties in creating the current anatomical TNM system is to review and rank all of the variable prognostic factors that have been published for a given site of cancer. In developing the sixth edition of the *AJCC Cancer Staging Manual (5)*, at least 80 prognostic factors for breast cancer were identified and assessed for their importance in the survival of patients with breast cancer. The strategy is to apply well-tested evidence-based methodology to each factor to assess the significance and statistical power prior to applying the specific factor to the TNM lexicon. The traditional prognostic parameters for human breast cancer have stood the test of time and currently include specific tumor factors of size, lymph node status, and grade of tumor. Other important factors such as lymphatic and vascular invasion and hormonal markers (estrogen or progesterone receptors) are certainly relevant, but have not yet been added to the official TNM status of breast cancer. Additional factors such as DNA content (ploidy, s-phase) have been considered important, but as yet have not been added to traditional TNM.

Along with tumor factors, it is important to consider factors specific for the individual patient or host. The patient age and menopausal status have of course been shown to be important as they specifically relate to the role of hormonal markers. Currently, the only site for anatomical

staging which includes age as a specific staging factor is thyroid cancer which indicates that patients under the age of 45 have a more benign form of their disease. In addition to the host factors of age and reproductive history, familial history and genetics will continue to be important as prognostic factors, but have yet not been added to traditional TNM. Other factors such as immune status and obesity also have a role to play in the prognosis of cancer. It is presumed that these two factors will be added to conventional anatomical staging as methodology improves to stratify patients with both immune and nutritional issues.

The role of axillary nodal and lymphovascular involvement cannot be overemphasized in the prognosis and staging of many solid tumors. Current strategies have included the number of tumor-containing nodes in the locoregional area as well as the volume of tumor contained in these nodal elements. Strategies to stage breast cancer and decisions for treatment of breast cancer are directly related to the microscopic involvement of lymph nodes in the axillary region. One of the important issues is to stratify nodes either into regional or distant sites and to address the issues of whether nodal involvement in a distant site is more important than locoregional nodal positivity. These decisions are especially important since clinical trials may well be based on the inclusion only of patients with locoregional disease while excluding patients with node positivity in distant sites. This became especially pertinent in women with breast cancer who had supraclavicular nodal disease. In the fifth edition of the AJCC and UICC staging strategies *(6,7)* these women were classified as having M1 or stage IV breast cancer. Review of larger data sets, however, showed that women with supraclavicular metastatic disease had the same survival as women with aggressive axillary disease and, therefore, should not be designated as stage IV based on supraclavicular disease alone. These patients have now been reclassified since 2002 as having N3 disease and, most importantly, are judged appropriate for clinical trials dealing with locoregional disease alone.

In the era of sentinel node evaluation, it has become especially appropriate to develop language within the TNM system that will allow for other nodal areas to be included such as the internal mammary chain. While these nodes are generally not assessed pathologically, they may be evaluated clinically utilizing modern computed tomography–positron emission tomography (CT–PET) studies and, therefore, should be included in the clinical portion (cTNM) of the TNM staging strategy. The finding of positivity in the internal mammary chain is especially relevant during discussion of radiation fields as an adjunct to mastectomy and chemotherapy in the breast cancer patient. A variety of studies have shown that internal mammary positivity itself is no worse as a prognostic factor, but that the combined positivity in both the axillary and internal mammary areas are associated with a worse outcome.

It is important in the recording of both clinical and pathological data relative to patients having sentinel node evaluation that there is a descriptor indicating that the sentinel node may be the only nodal entity examined. With this in mind, the suffix (sn) has been introduced to indicate that the lymph node whether negative or positive is a sentinel node only and does not indicate a node from a complete axillary or regional dissection. With the introduction of sentinel node evaluation in breast cancer, melanoma, head and neck cancer, and several GI sites, the importance of designating specific nodes in data collections is becoming more critical.

The assessment of nodal disease in staging strategies should not be taken out of context, but always should be associated with other variables relative to specific tumors. An example of this is melanoma of the skin in which thickness and ulceration of melanoma are specific and independent prognostic factors. The mitotic rate of the primary melanoma has also been found to have some important significance in prognosis and will most likely be included as a specific factor in staging of melanoma in the seventh edition of TNM *(8)*. The important issue is that the thickness of the melanoma (Breslow classification) and the appearance of microscopic ulceration are both surrogates of nodal involvement and metastatic behavior. As was true in breast cancer, the number of nodes involved with melanoma becomes an important prognostic variable. In addition to microscopic involvement of nodes, macroscopic nodal involvement indicated by clinical palpation is an important factor. Survival patterns in

melanoma generally worsen as the number of nodes involved increases in the locoregional draining areas of the primary melanoma. The total staging strategy of melanoma which depends upon the thickness, ulceration, and nodal involvement creates a dynamic staging strategy indicating that a nonulcerated melanoma with one microscopic node involved has a 63% 10-year survival versus a melanoma with ulceration and greater than four macroscopic (clinically palpable) nodes which has an 8% 10-year survival. (Fig. 1) It is obvious because of the capricious nature of melanoma that overall survival is not limited to 5 or even 10 years. Death resulting from melanoma continues to 15 years or more and is based on the biology of the tumor (Fig. 2). This is another example of the intertwining of overall cancer biology with traditional TNM anatomical staging.

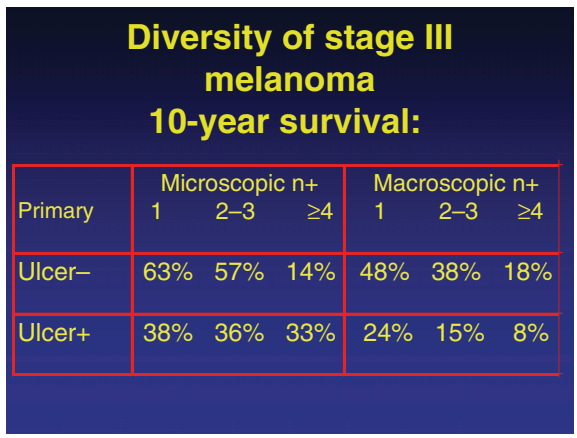


Fig. 1. Survival in melanoma based on tumor thickness and ulceration.

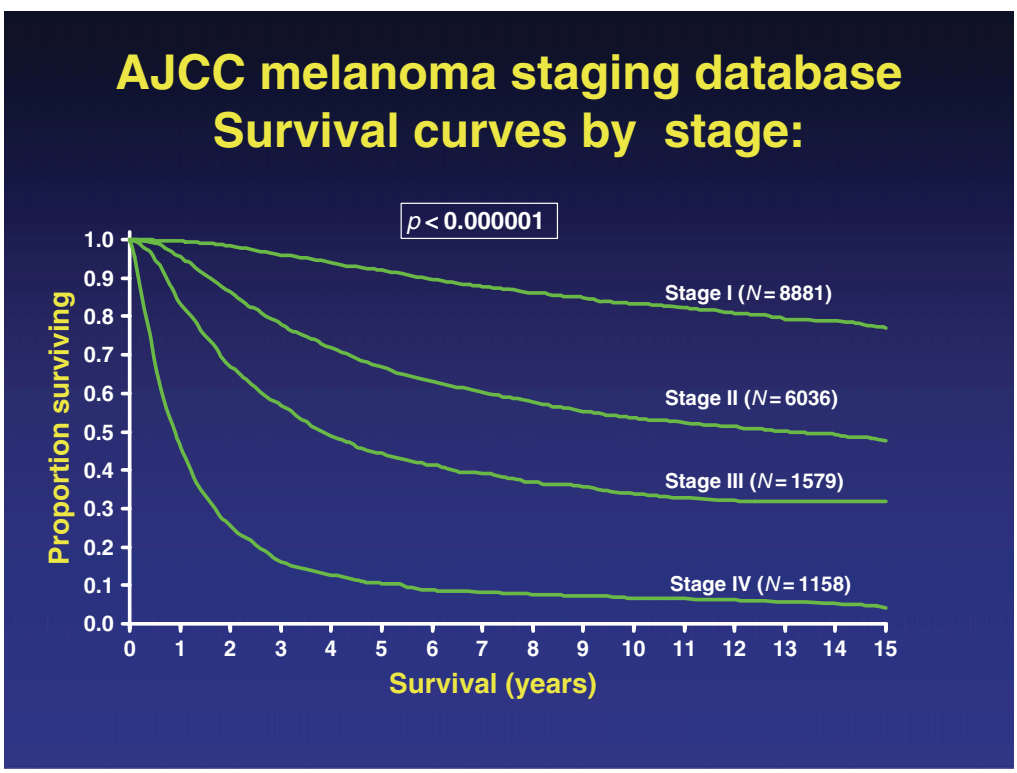


Fig. 2. Fifteen-year survival of melanoma patients based on TNM stage group.

An additional biological parameter is the concept of finding isolated tumor cells (ITCs) in lymph nodes. In contrast to micrometastases, the ITCs are defined as a cell or cluster of cells less or equal than 0.2 mm in greatest diameter *(9)*. These ITCs are indicated by the suffix (i+) or (i–) which is related to the specific nodes under study. Because of the improvements in both light microscopy with conventional stains and immunohistochemistry, smaller and smaller tumor deposits have been identified in the subcapsular and parenchymal regions of draining nodes. The finding of ITCs becomes extremely important when sentinel nodes are being evaluated which may only contain these small foci of tumors. While traditionally the ITC has been defined as a single cell or small cluster of cells that are less or equal to 0.2 mm, when multiple sites of small clusters are identified in the same lymph node, this obviously may have a much different prognostic implication and current opinions favor avoiding specific size constraints when dealing with ITCs *(10)*. The implication for treatment when only ITCs are found in lymph nodes is important especially since traditional TNM staging continues to define this category of nodal involvement as N0. There is evidence that indicates that the discovery of ITCs in the absence of nodal micro- or macrometastases may have adverse prognostic significance in melanoma *(11)*. The ITC concept will obviously become more important as earlier stage disease is found and more aggressive sentinel node examination is undertaken.

While it is difficult for medical oncologists to recommend against systemic chemotherapy when ITCs have been found in a patient's regional nodal bed, it is important that clinical trials be developed that will answer the questions relative to the prognostic implication of minimal nodal positivity. This is especially true in the era of molecular diagnostics in which polymerase chain reaction (PCR) and direct measurement of tumor RNA become more commonly utilized. This molecular staging strategy has necessitated the creation of a suffix (mol) which indicates either a positive or negative finding in the lymph node when PCR is applied. It is anticipated that most institutions in the future will have the technology to easily perform molecular diagnostic testing on nodal tissue. This approach has now been introduced for the intraoperative assessment of sentinel nodes in the management and decision making for breast cancer *(12)*.

Previous iterations of the TNM system tended to lump patients with nodal positivity into broadly defined staging groups. This was especially evident in the classification of colorectal cancer where patients with positive mesenteric nodes were stratified to a common group collectively designated as stage III. Utilizing large data sets provided by the National Cancer Data Base (NCDB) it became evident that three specific subsets of patients could be defined based on depth of penetration of the primary tumor into the wall of the bowel and the number of nodes found to be positive in the mesentery *(13)*. These three subsets defined as stages IIIA (T1-2, N1), IIIB (T3-4, N1), and IIIC (any T, N2) showed three characteristic and distinct survival curves (Fig. 3). The implication is important in that it may mean that the stage IIIA patient should be treated in a different fashion and with different chemotherapeutic agents than the stage IIIC patient. Currently most patients with node-positive colon cancer receive similar chemotherapeutic agents and the treatment stratification according to subsets of stage III disease has not been instituted.

The corollary to the staging of colon cancer is that an adequate number of nodes must be resected by the surgeon and investigated by the pathologist in order to adequately assess each patient. Current data suggest that there is a more favorable outcome for patients based on the ability to resect and identify a greater number of nodes regardless of pathological findings (Fig. 4). The ratio of positive to total nodes resected is also being studied and may be an important prognostic factor in gastrointestinal as well as other sites. The total number of nodes resected may have an even greater benefit in stage II patients in which positive nodes have not been identified. This has become apparent by examining a large group of stage II patients reported in the NCDB. The number of nodes resected in colon cancer is currently being used as a benchmark of quality for both surgeons and pathologists and will ultimately have implications for payment strategies to individual practitioners as well as institutions.

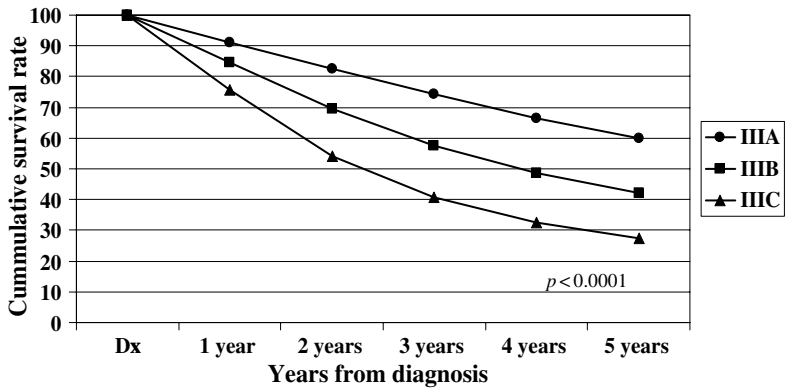
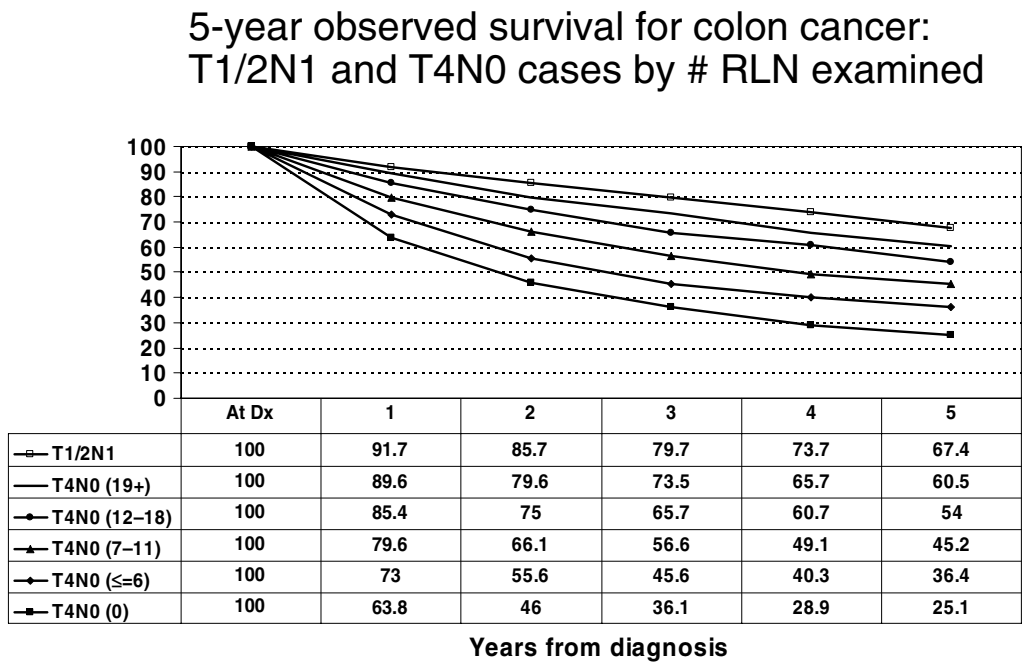


Fig. 3. Five-year survival of subsets of stage III colon cancer.



	At Dx	1	2	3	4	5
T1/2N1	100	91.7	85.7	79.7	73.7	67.4
T4N0 (19+)	100	89.6	79.6	73.5	65.7	60.5
T4N0 (12–18)	100	85.4	75	65.7	60.7	54
T4N0 (7–11)	100	79.6	66.1	56.6	49.1	45.2
T4N0 (≤=6)	100	73	55.6	45.6	40.3	36.4
T4N0 (0)	100	63.8	46	36.1	28.9	25.1

Fig. 4. Survival patterns as a result of decreasing number of nodes in T1-2, N1 and T4N0 colon cancer (from the National Cancer Data Base).

There are other sites included in TNM anatomical staging which take account of nodal assessment, but which do not fit easily into the strategies previously described in breast, melanoma, and colorectal cancer. One of these is soft tissue sarcoma in which nodal involvement is an uncommon finding and has a more grave prognostic implication when found. Specific histological types of sarcomas may commonly involve regional nodes. These include synovial sarcoma and embryonal rhabdomyosarcoma. Any nodal involvement in sarcoma is considered to be similar to visceral metatstatic disease and, therefore, is given a stage IV designation. The biology of sarcoma is much more dependent on histological grade and depth of penetration especially in the types of sarcoma in which nodal disease is not a frequent characteristic.

Unlike sarcoma, in differentiated thyroid cancer (papillary or follicular), nodal involvement may have a very benign course. While it is becoming more fashionable to resect nodes in the management of differentiated thyroid cancer, large data sets show that nodal positivity may be associated with significant long term survival. As stated previously, the age of the patient and the primary histological variant of tumors of the thyroid are more important as prognostic factors than even nodal involvement. Specific areas in the neck especially the central region (level VI) should be assessed in the management of thyroid tumors. Specific nodal levels in head and neck cancer have prognostic implication especially with tumors of the oropharynx and larynx. Traditionally, these nodal sites have been resected during radical procedures for head and neck tumors. These regional nodal beds are also used as diagnostic clues for localization in patients who have occult tumors of the head and neck.

Staging strategies are thus dependent on prognostic factors that involve the primary tumor, the patient and even the environment as it relates to the opportunities for early treatment and follow-up care *(14)*. Newer and more specific prognostic factors dealing with molecular diagnostic studies are being introduced into staging strategies. In the future the traditional anatomical staging will be closely linked with molecular markers. The T, N, and M descriptors along with other prognostic factors will be primary data points in nomograms relating to a number of tumor sites *(15,16)*. These data elements will be fed into either handheld or internet-based sites in order to give direction for both patients and physicians and to help with decision making relative to the type of multidisciplinary care that is necessary for treatment. All of these together will add up to a prognostic quilt that will have a somewhat different appearance than our traditional anatomical concepts. The future depends on newer diagnostic methods which are being introduced into pathological assessment and more importantly into clinical and preoperative imaging. The traditional dichotomy of clinical staging (cTNM) and pathological staging (pTNM) must be melded into one continuum and should allow for the interaction of both clinical and pathological elements in our language of staging. All of this will depend on improved data collection and especially the science of medical informatics which is based on the collection of large data sets. The data acquisition for melanoma and gastrointestinal cancer has been utilized to refine our traditional staging strategies and this concept will no doubt be used in other sites as we go forward with the blending of anatomical and molecular markers.

The introduction of artificial intelligence and the unification of concepts within the structure of nomograms will no doubt help to refine the language of cancer and will give a greater acuity of information to physicians, patients, and their caregivers. The one factor that still remains relatively undefined and elusive is the understanding of the individual biology of the tumor. This is the real Holy Grail of staging and we have yet to comprehend the specific biological factors that will give us the ultimate prognostic information. As Sir William Osler stated in the early twentieth century, "Medicine is a science of uncertainty and an art of probability."

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IX Immune Responses in the Draining Lymph Nodes Against Cancer: Implication for Immunotherapy

33 Role of Lymph Nodes in Immunotherapy of Malignant Tumors

Walter T. Lee, MD and Suyu Shu, PhD

CONTENTS

ABSTRACT

The tumor-draining lymph node (TDLN) is the site of critical immune interactions and has implications in the clinical outcome of patients with malignant tumors. This chapter reviews the anatomy of lymph nodes and the interactions that occur between tumor antigens and immune cells at the TDLN. Historical in vivo experiments demonstrating the role that lymph nodes have in the immune response to malignancies is presented. These findings form the basis for ongoing research to develop approaches that potentiate the antitumor immune response. We conclude by describing some of these approaches. This includes the generation of effector cells from TDLN for adoptive immunotherapy and a dendritic cell–tumor fusion hybrid vaccine that demonstrates efficacy when delivered directly into lymph nodes.

Key Words: Immunotherapy; lymph node; malignancy; fusion hybrids

1. INTRODUCTION

The tumor-draining lymph node (TDLN) is the site of dichotomy with respect to tumor metastases and critical immune interactions and has significant clinical implications. Indeed, the status and extent of lymph node involvement for a progressive tumor is one of the three factors used by American Joint Commission on Cancer (AJCC) tumor–node–metastasis (TNM)

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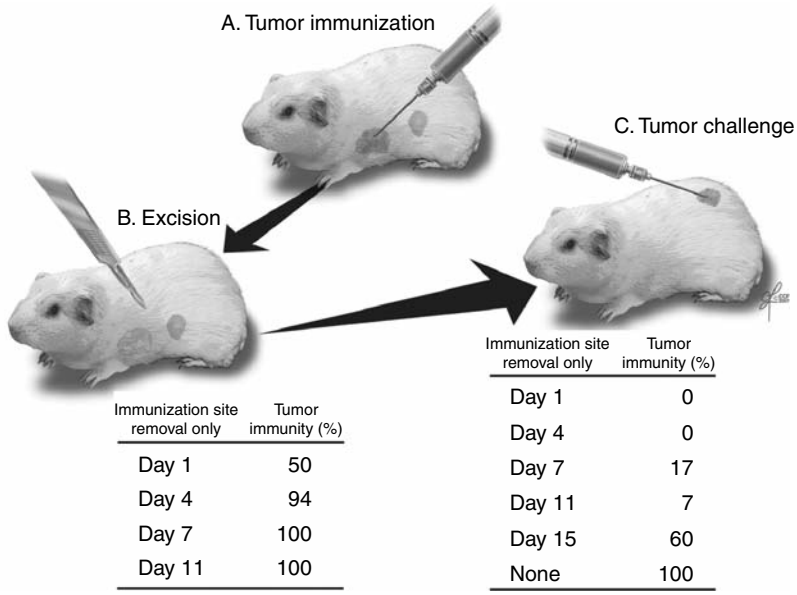
staging system and is a biological indicator of tumor spread. The presence of lymph node involvement for many malignancies is automatically considered advanced stage (III or IV) such as melanoma, colon carcinoma, and head and neck squamous cell carcinoma. In fact, the AJCC has recently revised and incorporated the most important predictors of survival in nodal disease (number of metastatic lymph nodes and microscopic or macroscopic tumor burden) in its current cutaneous melanoma staging system *(1)*. Although the TDLN is the first site of early metastasis, it is this lymph node where antitumor immune responses can be initiated. Presentation of tumor antigens and products at this site can generate tumor-reactive effector cells. Conversely, the spread of tumor to lymph nodes may indicate a deficient immune response against the malignancy. Because the majority of solid tumors are involved with the lymphatic system, understanding the pivotal role that these lymph nodes have in immune stimulation is critical to successful cancer immunotherapy.

2. STRUCTURE AND ANATOMY

The lymph node is a dynamic environment and critical for eliciting an immune response. It is comprised of a reticular support network surrounded by a thick capsule pierced by afferent lymph vessels. Three areas are identifed within the superficial lymph node architecture: the cortex, paracortex, and medulla. The cortex contains B cell enriched follicles. The paracortex contains high endothelium venules (HEV) that function as a location for specific T lymphocyte migration from blood to the lymph node parenchyma *(2)*. HEV are where naive T cells encounter mature dendritic cells or other antigen-presenting cells (APCs) actively presenting antigens. The medulla is comprised of cords and sinuses containing macrophages and activated T and B cells. Immune cells leave the medulla sinuses via the efferent lymph vessel. Recently developed imaging techniques for in vivo tracking of cellular interactions allow investigators to observe the presentation of tumor antigens to T cells and the proliferation of these specific T cells in the TDLN *(3–5)*. These recent in vivo observations support the fact known to tumor imunologists for decades, that specific antitumor effector T cells with therapeutic effects can be generated from TDLN.

3. GENERATION OF EFFECTOR CELLS FOR ADOPTIVE IMMUNOTHERAPY

The importance of the TDLN in tumor immune response was demonstrated in an elegant experiment using the Line-10 hepatoma in strain 2 guinea pigs *(6)*. Hanna et al. prophylactically immunized guinea pigs with irradiated Line-10 hepatoma (1×10^6) admixed with *Mycobacterium bovis* strain bacillus Calmette–Guerin (BCG) as an adjuvant. This immunization would protect against a subsequent, otherwise lethal, tumor challenge. In order to demonstrate the role of the TDLN in tumor immunity, at predetermined time points, the immunization site was surgically removed. In some animals, the TDLN was also removed along with the immunization site. Guinea pigs were then challenged with a lethal dose of Line-10 hepatoma to test for the development of systemic immunity. Animals in which the immunization site alone was removed within 4 days were able to demonstrate significant immunity against subsequent tumor challenge. However, the concomitant removal of the TDLN would result in abrogation of the antitumor immune response. In fact, animals in which both the immunization site and the TDLN were removed as late as day 15, the development of a significant systemic immunity would be impeded. These observations indicated that a critical period of an intact TDLN is necessary for the development of systemic antitumor immunity. Furthermore, these findings suggest that critical immune sensitization occurs within the lymph node rather than at the primary tumor site (Fig. 1).



Immunization site removal only	Tumor immunity (%)
Day 1	50
Day 4	94
Day 7	100
Day 11	100

Immunization site removal only	Tumor immunity (%)
Day 1	0
Day 4	0
Day 7	17
Day 11	7
Day 15	60
None	100

Fig. 1. (**A**) Guinea pigs were immunized using Line-10 hepatoma and BCG. (**B**) At a set time point, the immunization site alone or immunization site with the TDLN were excised. (**C**) Animals were then challenged with an otherwise lethal dose of Line-10 to check for immune protection. Animals in which the immunization site alone was removed demonstrated systemic immunity if excised after 4 or more days. Animals in which the immunization site with the TDLN demonstrated systemic immunity if excised after 15 days. This suggested that a critical time period for an intact TDLN is needed to generate systemic immunity.

These historical experiments demonstrated that the TDLN was critical to development of an antitumoral response. However, it is clinically known that patients with tumor metastasis to the TDLN represent a poor prognosis. On the other hand, immunotherapy research is directed towards harnessing, activating, and expanding effector cells from TDLN while overcoming suppresive elements that prevent an effective antitumor response. In the early 1990s, an in vitro culture method was developed that involved culturing and expanding effector cells from TDLN for subsequent therapeutic usage. This therapeutic approach is often referred to as adoptive immunotherapy.

A typical preclinical adoptive immunotherapy protocol involved the following steps. TDLN are harvested from animals bearing subdermal tumors. TDLN cells are then nonspecifically activated in vitro for 2 days with immobilized anti-CD3 antibody. This stimulation serves to induce the expression of high-affinity IL-2 receptors on T cells and proliferation of tumor-sensitized T cells. Cells are then expanded with low doses of IL-2 (4 U/ml) for an additional 3 days. Cells are then harvested and prepared for systemic transfer into tumor-bearing animals *(7,8)*. The procedure is illustrated in Fig. 2.

An example of an experimental treatment model involves the treatment of pulmonary metastasis. Pulmonary metastasis can be easily established by injection into the tail vein thereby seeding the lungs. After 10 days, activated effector cells (50×10^6) are transferred intravenously to these animals. Untreated animals routinely have >250 metastatic lesions on the lung surface after 3–4 weeks. Mice adoptively transferred with effector cells significantly reduced the lesions seen *(9)*. Despite the nonspecific nature of the in vitro anti-CD3/IL-2 activation, adoptive therapy was exquisitely specific for the tumor that stimulated TDLN in vivo. Furthermore, the anti-CD3/IL-2 method resulted in an eightfold proliferation of these tumor-specific effector T cells.

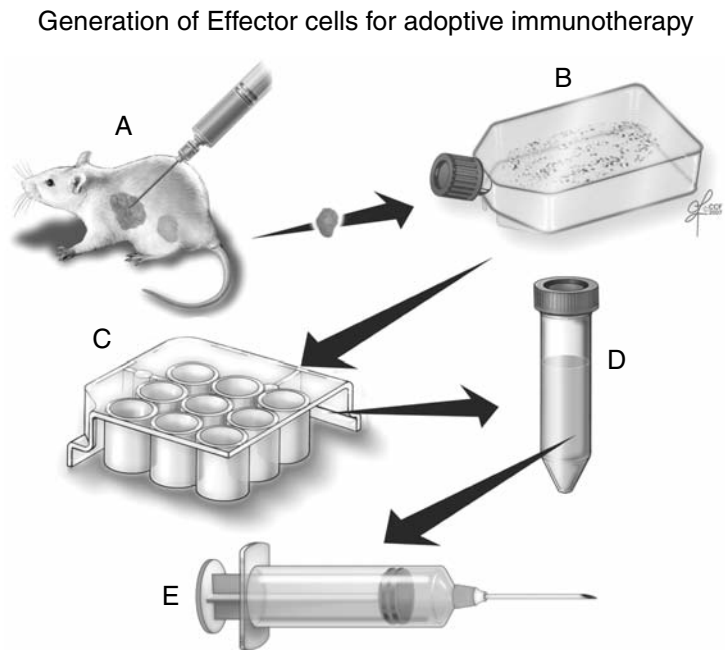


Fig. 2. (**A**) Animals are inoculated with tumor. As the tumor grows, the TDLN is harvested and made into a cellular suspension. (**B**) These cells are then cultured with anti-CD3 for 2 days. (**C**) Cells are then recultured with IL-2 for an additional 3 days. (**D**) Activated cells are harvested. (**E**) These cells are then injected intravenously into the host.

These and other similar findings led to using adoptive immunotherapy in clinical trials involving melanoma, renal cell carcinoma, head and neck squamous cell carcinoma, and malignant gliomas. After tumor was surgically obtained from the primary site, cells were cultured in attempts to establish tumor lines. Tumor cells were inactivated by irradiation and injected intradermally at the anterior upper thigh along with granulocyte macrophage colony-stimulating factor (125 μg) as an adjuvant. This area drains directly to the inguinal lymph nodes. These TDLN were then harvested approximately 10 days after inoculation and cells were activated and proliferated using the anti-CD3/IL-2 or SEA/IL-2 method. Effector cells after 10–14 days of culture were then tranferred back into the patient. Although the cell transfer was well tolerated and a small number of patients with dramatic clinical response, there was a lack of significant responses for most of the patients *(10–13)*. Adoptive immunotherapy continues to be investigated in combination with other immune manipulations to improve its therapeutic efficacy *(13,14)*. We believe that for further development of antitumor immunity, antigen presentation to the immune system will need to be addressed and strengthened.

4. KEY ROLE OF ANTIGEN-PRESENTING CELLS

Tumor antigen presentation to T cells is a key component of successful immunotherapy. Over the past 20 years, it has been shown that the most potent APC known is the dendritic cell (DC). Their key role in regulating T and B cell immunity are due to a number of characteristics. DCs exhibit high levels of MHC class I and II molecules, and costimulatory molecules (i.e., B7 family) *(15)*. Cytokines secreted by DC, such as IL-12 and IL-6, result in growth of cytotoxic and helper T cells *(16,17)*. In addition, DCs also have the ability of cross-priming CD8+ T cells *(18,19)*.

The maturation stage of DC is important in immune regulation. Immature DCs (iDCs) do not express high levels of MHC class I and II molecules and have been found to induce immune tolerance *(20,21)*. Although antigen uptake is enhanced in iDC, activation and maturation of these DCs are necessary for upregulation of costimulatory molecules, expression of chemokine receptors, and enhancement of antigen presentation function. Maturation is also important in DC migration. Once antigen uptake occurs, maturation and migration of DC result in antigen presentation to T cell in the lymph node. Studies in the TDLN of melanoma patients have shown that mature DCs migrate and are present in the TDLN. Conversely, iDCs are observed not in the TDLN but rather the epidermis where antigens are encountered and uptake occurs *(22)*.

Based on the specialized ability of DC for antigen presentation, a number of immunotherapy strategies have been pursued. These efforts include DC pulsed with whole tumor lysate, transfection of DC with tumor DNA and RNA, and DC genetically engineered to produce tumor antigens *(23–30)*.

One promising strategy that utilizes the specialized properties of DC is cell fusion. Historically, whole tumor cells, inactivated by radiation or mitomycin-C treatment, were found to be the best antigens for immunization. The priniciple behind cell fusion is that the newly created hybrid cell has properties of both parental cells. Thus, fusion of DC and irradiated tumor cells should result in a new cell that uses the antigen-presenting properties of the DC to present all known and yet defined antigens from the tumor cell. Such a fusion hybrid should be potent in presenting antigens to both MHC class I- and class II-restricted pathways. Studies have found fusion hybrids to express a wider complement of both endogenously expressed tumor antigens and DC-expressed molecules linked to efficient antigen presentation when compared to other DC-based approaches *(31,32)*.

5. PROCESS OF DC–TUMOR CELL FUSION

One method of cell fusion involves exposing cells to chemical agents such as polyethelene glycol. However, we have found this method to have poor yields to lack consistently verifiable confirmation of DC–tumor fusion hybrids *(33)*. Alternatively, cell fusion can also be achieved by exposing cells to electric fields through a process called electrofusion.

The electrofusion involves two essential steps. The first involves the creation of an oscillating dipole on the cells by exposure to an alternating electric current (ac). This phenomenon is known as "dielectrophoresis." This results in the cells migrating toward the poles of nearby cells, creating a "pearl-chain" appearance. This alignment brings the cell membranes into close contact. The second step involves inducing a reversible membrane breakdown. A short direct electric current (dc) pulse is then delivered which disrupts the cell membrane at the points of cell to cell contact (Fig. 3). It is theorized that during this dc pulse, the phospholipid bilayer of cell membranes are disrupted. When the pulse is completed, and the phospholipids begin to randomly reorganize, fusion of adjacent cell membranes occur. This is depicted in Fig. 4. If the dc pulse is not strong enough, then fusion does not occur. If the dc pulse if too strong, then irreversible membrane occurs causing destruction of the cell membrane integrity.

In our laboratory, irradiated tumor cells are mixed with mature DC at a 1:1 ratio. They are placed into a custom fusion chamber designed to maximize the number of fusion cell yields. After exposure to the electrofusion process, the cells are gently harvested and cultured overnight. A variety of methods have been used to verify the electrofusion formation of DC–tumor hybrids. These include Geimsa stain cytospin, fluorescent-activated cell sorting analysis, confocal flourescent microscopy, and nuclear DNA content. Fusion rates of 20–40% have been achieved using a number of distinct murine tumors including SCCVII squamous cell carcinoma, D5LacZ (B16) melanoma, MCA 205 fibrosarcoma, and 4T1 breast carcinoma *(31,34,35)*.

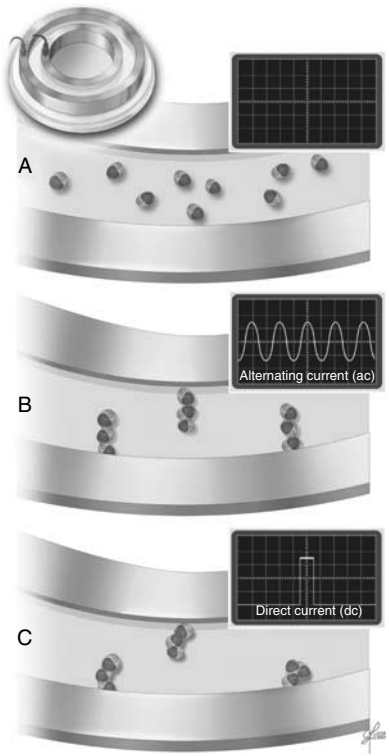


Fig. 3. The process of electrofusion. (**A**) Cells are suspended in a fusion chamber between two electrodes. (**B**) Application of an alternating current results in dielectrophoresis and "pearl chaining" of the cells. (**C**) Subsequent exposure to a short direct current results in reversible membrane breakdown and cell fusion.

6. SENSITIZATION BY FUSION CELLS

Preclinical therapy experiments using DC–tumor fusion cells were performed. For example, pulmonary metastasis were established in animals via tail vein injection of tumor cells. Three days later, fusion cells were administered and the number of lung metastasis were enumerated after 3 weeks of tumor progression (Fig. 5). In order to obtain therapeutic efficacy in these models, two conditions were found to be necessary. The first is the need for a third signal to be administered in conjunction with the DC–tumor fusion cells. Third signals include 4-1BB and anti OX-40L monoclonal antibodies (mAb), as well as IL-12 given intraperitoneally. Animals treated with DC–tumor fusion hybrids or third signal alone did not show a significant decrease in numbers of metastasis. However, when fusion cells were given along with a third signal reagent, significant therapuetic effects were observed (Fig. 6). The exact mechanism in which this third signal works is currently yet be be fully understood, and is the subject of investigations *(36)*.

Successful therapeutic vaccination also requires the direct delivery of the DC–tumor fusion cells into secondary lymphoid organs, such as the spleen or lymph node. This intralymphoid delivery of DC–tumor fusion cells resulted in both CD4 and CD8 antitumor activation. Both were demonstrated to be necessary for in vivo antitumoral effects *(31)*. Administration of fusion hybrids through other routes, such as subcutaneous or intraperitoneal, did not result in therapeutic efficacy. This aspect underscores the critical role that lymph nodes have in the sensitization of antitumoral effector cells. These two important findings have been confirmed in over 100 experiments involving fusion cells in multiple murine tumor models.

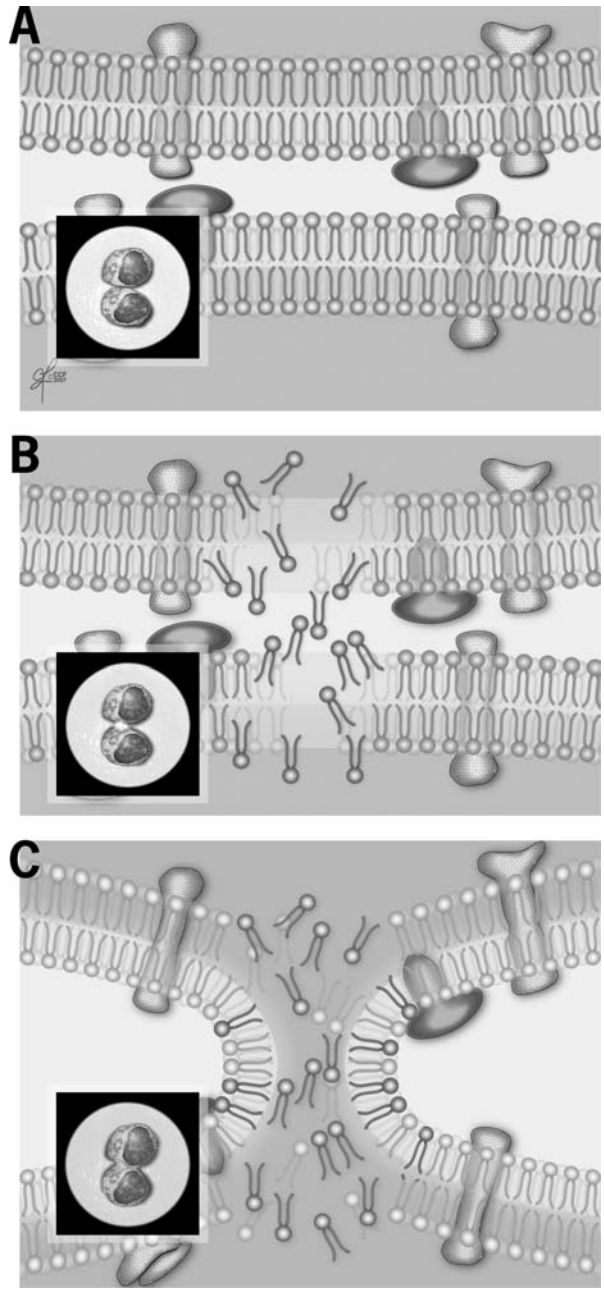


Fig. 4. Model of reversible membrane breakdown on a molecular level. (**A**) Cell membranes are brought into close proximity by the alternating current. (**B**) As the direct current is applied, the phospholipid bilayer of the cell membrane becomes disrupted. (**C**) At the completion of the direct current, the phospholipids mix and randomly rearrange such that the membranes of adjascent cells are now fused.

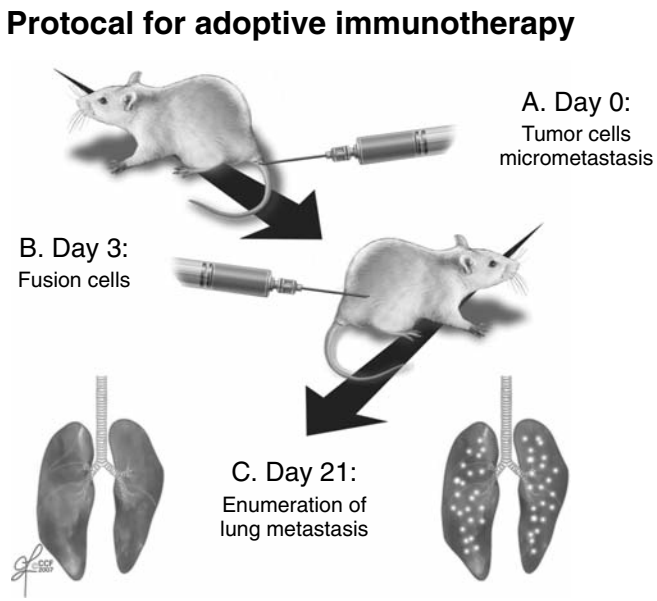


Fig. 5. A typical treatment model using DC–tumor fusion cells. (**A**) Tumor cells are injected in the tail vein to produce pulmonary metastasis. (**B**) DC–tumor fusion cells are injected into the lymph nodes with or without systemic third signal. (**C**) After 21 days, the number of pulmonary metastasis are enumerated.

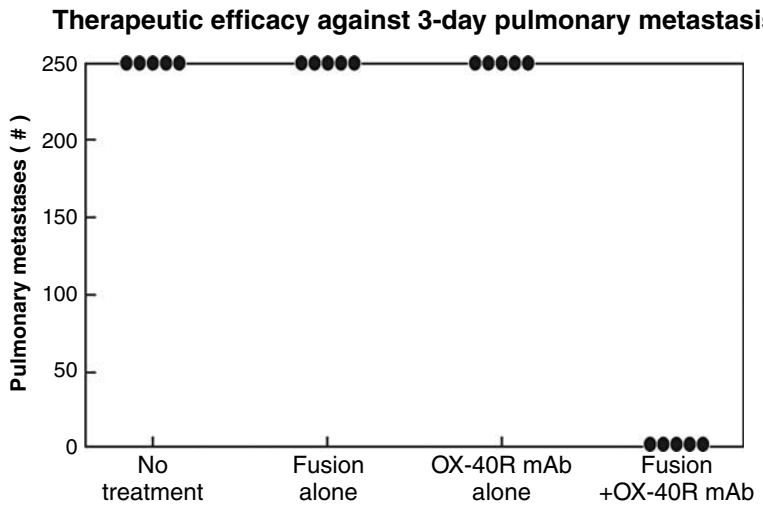


Fig. 6. A representative experimental result. Fusion cells or third signal alone (anti-OX-40R mAb) did not demonstrate differences from untreated controls. However, the combination of fusion cells and third signal did result insignificant therapeutic results.

7. FUTURE DIRECTIONS

Fusion cells have thus far utilized syngeneic tumors as the tumor fusion partner. Clinically, the use of autologous tumor will involve obtaining a sample from each patient, establishing a cell line, and then determining the optimal fusion conditions. This process will be labor intensive,

time consuming, and unpredictable. It is known that tumors from the same histological origin share antigens known as tumor-associated antigens (TAA). These TAA may serve as immunotherapy targets. TAA are best characterized in melanoma with antigens such as gp-100, tyrosinase, MART-1, and melan-A. Currently, we are investigating the use of allogeneic tumors as fusion partners in hybrids that share TAA with the target tumor.

The use of a third signal is also being investigated for clinical use. DC culture methods may be able to mature and stimulate them to provide enough third signal, thus obviating the need for concurrent administration. Alternatively, research is ongoing to identify other third signals that would be better tolerated by patients.

The role of lymph nodes in tumor immunology is becoming better understood and more appreciated. The interactions among DC that occur within the dynamic lymph node environment are critical to activating effector cells against malignant tumors. It is known that tumors can induce immunosuppression and these can be demonstrated in the TDLN. Thus, any vaccine design should not only increase effector cell sensitization but also decrease the immunosuppression factors present in the environment. Development of immunotherapy strategies should consider the critical roles that lymph nodes have in generating a clinically effective antitumoral response.

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34 Tumor-Related Immune Modulation of the Regional Lymph Nodes

Rong–Rong Huang, MD, Eijun Itakura, MD, PhD, Duan-Ren Wen, MD, and Alistair J. Cochran, MD

CONTENTS

ABSTRACT

Development of nodal or visceral metastases in patients with cutaneous melanoma correlates with the thickness and depth of the primary tumor. Locoregional metastases first affect the sentinel node (SN), the first lymph node(s) on the direct lymphatic drainage pathway from a primary melanoma. Downregulation of SN function may due to bioactive molecules released by tumor cells.

We report findings from our own research and reports of other investigators that related to tumor–lymph node interactions.

Individual lymph nodes in a regional nodal group react heterogeneously, with nodes located close to the primary or nodal metastatic tumor being least reactive and most downregulated. SNs have been found to be relatively downregulated compared to nonsentinel node (NSN). SNs show alterations of cellular activity within the paracortex, including alterations of antigen-presenting dendritic cells, and the frequency of dendritic cell-associated activated T cells. Endothelial cells of high endothelial venules in SN show alteration in activation markers even before there is evidence of development of metastases. In the SN such endothelial cell are flatted and there is reduced transendothelial migration of activated T cells when SNs are compared to NSNs. From studies of animal models of melanoma and some finding in breast cancer patients, metastasis-free SN paracortical venules are dilated, demonstrating a large lumen and thin walls with an apparent shifting of their primary function from lymphocyte recruitment to blood transportation and distribution. Blood vessel enrichment of SN is seen before metastases occur.

Molecular studies have demonstrated that SN immune modulation is due to the influence of highly active tumor-derived molecules such as interleukin (IL)-10, interferon (IFN)-γ, indoleamine 2,3 dioxygenase (IDO), and transforming growth factor (TGF)-β. Treating melanoma patients with granulocyte-macrophage colony-stimulating factor (GM-CSF) or cytosine–phosphate–guanine oligodeoxynucleotides (CpG) prior to SN biopsy have shown that SN downmodulation is susceptible to therapeutic reversal.

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We hypothesize that tumor-induced downregulation of SN function may be the basis of susceptibility of SN to the survival and progression of the tumor cells that are the source of clinically significant metastases. Locoregional therapy may influence the evolution of locoregional and systemic metastases and thus clinical outcome.

Key Words: immunity; lymph nodes; immune suppression; melanoma; metastases

The following comments are based on talks given by A.J.C. and R–R.H. at the 1st and 2nd International Symposia on Cancer Metastasis and the Lymphovascular System (April 2005, May 2007) in San Francisco. They represent expanded summaries of these talks with the addition of more contemporary findings and data from the literature. The chapter draws on previous publications from our laboratory *(1–9)*.

Most patients with cutaneous melanoma never develop nodal or visceral metastases. This is particularly true of patients with thin (<1 mm thick), superficially located primary melanomas that have been completely excised with a margin of normal skin. Patients who do develop metastases usually have thicker (≥1 mm) and more deeply invasive primary melanomas and such patients most often first develop metastases that are detectable by palpation or ultrasound in the ipsilateral regional nodes *(10)*. The earliest stages of locoregional metastases preferentially affect the sentinel node (SN), the first lymph node (or nodes) on the direct lymphatic drainage pathway from a primary melanoma or other solid tumor *(11)*. This may simply reflect the flow dynamics of lymph that travels from the area of the primary tumor to the SN and that may transport viable and metastasis-competent tumor cells that are shed from the primary tumor. Such cells must have the capacity to survive, proliferate, and establish metastatic colonies in the tissues of lymph nodes. Alternatively, the SN may be selectively permissive of colonization by and expansive growth of metastatic tumor cells. This would imply that in the SN the mechanisms characteristic of "normal" lymph nodes that operate to prevent the survival and proliferation of extraneous cells, including tumor cells, are downregulated. There is interest in the possibility that SN downregulation may be induced by molecules generated and released by tumor cells that can affect the functional competence of lymphoid and other cells in the lymph nodes *(6,7,12,13)*.

Prior to our development of the techniques of lymphatic mapping and SN biopsy that permit the identification and assessment of the SN *(14)*, we undertook a series of studies of lymph nodes oriented according to their proximity to a primary tumor (melanoma or breast cancer) or to lymph nodes colonized by metastatic tumor. We demonstrated that individual lymph nodes within a regional node group were heterogeneous in the extent of their reactivity. Indices examined included microscopically determined reaction patterns (paracortical hyperplasia, follicular hyperplasia, and sinus histiocytosis), frequency and density of S-100-positive paracortical dendritic cells (PDC) and the frequency, length, and complexity of their dendritic processes *(15)*, T cell activation, the capacity of T cells to blast transform when exposed to phytomitogens or transplantation antigens *(16)*, spontaneous production of migration-inhibiting lymphokines *(17)*, cytotoxicity against melanoma cells in culture *(18)*, and the frequency of ConA-inducible suppressor cells *(19)*. In all such studies the nodes that were least reactive and thus most downregulated were located close to primary or nodally metastatic tumor. These studies were inherently limited by the fact that we could not be certain that a specific node, even if located relatively close to tumor, actually received lymph through lymphatics that derived from the environs of primary or metastatic tumor. The development of lymphatic mapping and SN biopsy, for the first time allowed us the possibility of studying the immunobiology of a node that we knew received lymph and its molecular and cellular contents that originated from or in the immediate vicinity of a primary tumor.

We therefore extended our initial anatomy-based studies to compare the distribution, cytology, and cellular phenotype of cell populations in metastasis-susceptible SN with those in more remote nonsentinel node (NSN) from the same melanoma patient *(1,2)* or breast cancer *(3)* patient . Compared to NSNs, SNs are entirely or segmentally *(4)* downregulated as evidenced by a reduction in the aggregate area of the paracortex, the area of the node occupied by PDC, the frequency, density, meshworking, and dendritic complexity of PDC *(5)*. Alterations in the SN thus affect the critical antigen transporting and presenting dendritic cells that migrate to the node from peripheral tissues including tumor and tumor-associated skin. Also affected are the T lymphocytes that under normal circumstances are the clients of the antigen-presenting cells (reduced T cell area within the nodes, reduced density of T cells, reduced expression of activation markers, and reduced T cell migration through the walls of high endothelial venules [HEVs]) *(6)*. As a result of this immune suppression there is a reduction in the availability of continuing supplies of tumor-directed cytotoxic $CD8^+$ T cells, cells that are viewed as having a major role in limiting local evolution of the primary melanoma and inhibiting the establishment and expansion of metastases. In this way nodal dysfunction has the potential to exert a significant influence on tumor cells in visceral sites remote from the regional nodes. Thus, contrary to the opinion held by some, locoregional therapy may very well influence the evolution of systemic disease and clinical outcome.

Since naïve T lymphocytes arrive for their encounter with antigen-presenting dendritic cells by migrating across the endothelium of HEVs in the paracortex, we examined the vascular anatomy of SN for differences in venular pattern, frequency, and endothelial activation relative to NSN. There is a reduction in the height of their endothelia in SN paracortices and an increased coefficient of variation of HEV frequency segmentally in SN compartmentalization prior to there being evidence of nodal metastases. There is also a reduction in transendothelial migration of T cells and an apparently associated reduction in the frequency of PDC-associated activated T cells *(7)*. Qian et al. *(13)* used an animal model and human breast cancer to study the basis of lymphangiogenesis and angiogenesis within SN. They found that compared to noncancerous control lymph nodes blood vessels in metastasis-free SN from breast cancer patients were dilated with a large lumen and thin walls, shifting their primary function from recruitment of lymphocytes to transport and distribution of blood. They concluded that the SN is "rebuilt" by the primary tumor to become a functional blood vessel-enriched organ before and independent of the development of metastases. Tumor induced vascularization can thus be a regional process rather than purely a local event.

Vuylsteke's group demonstrated that immune function of SN was suppressed compared to NSN and that administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) around the excision site of the stage I primary melanoma increased the number and activation state of dendritic cells in the paracortical areas of SN and enhanced dendritic cell binding to T cells *(20)*. Lee et al. also provided molecular evidences of cytokine-induced SN immune suppression that was associated with increased interleukin (IL)-10, interferon (IFN)-γ, and indoleamine 2,3 dioxygenase (IDO), considered to be generated by the cells of the primary melanoma in SN *(21)*. Immune downregulation of the SN and its potential reversal have been confirmed by other investigators *(22–27)*.That the alterations noted in SN are not fixed is clearly demonstrated by these observations that they are not present in the SN of patients treated preoperatively with intradermal peritumoral GM-CSF *(20,28,29)* or cytosine–phosphate–guanine oligodeoxynucleotides (CpG) *(30)*. Such observations strongly support the view that SN immune modulation is tied to the influence of tumor in the catchment area of the node, and is capable of therapeutic reversal. Selective downregulation of the SN may be induced by suppressor cells, but certainly involves tumor-derived products such as gangliosides *(31,32)* and cytokines including IL-10 *(8,26,33)* and transforming growth factor (TGF)-β *(27)*.

IL-10 is a highly immunosuppressive cytokine. In patients with residual melanoma at the primary site or a tumor-positive SN, IL-10 expression level is higher in SN than NSN *(21)*. TGF-β is also powerfully immunosuppressive, but additionally, has dual tumor-suppressor and tumor-promotor effects on tumor cells *(34)*. Melanoma cells are reported to be generally resistant to the tumor-suppressive effects of TGF-β *(35)*. Immunosuppressive factors are secreted by inflammatory cells infiltrating the tumor as well as by the tumor cells themselves *(36)* and readily transported in the afferent lymph to the SN. Vascular endothelial growth factor (VEGF), an important growth factor for endothelial cells, plays a major role in tumor angiogenesis *(37,38)*. VEGF may have an additional role in recruiting monocytes from the bone marrow to the primary tumor site. Such monocytes are "educated" in the tumor microenvironment and evolve into tumor-associated macrophages (TAM) *(39)*. TAM in turn produce angiogenic factors such as VEGF and IL-8 and thus contribute to tumor angiogenesis *(40)*. TAM also have immunosuppressive characteristics and release immunosuppressive factors into the tumor microenvironment *(39)*. TAM and their products are known to travel via the afferent lymphatics and thus can affect the immunological competence of SN.

Our findings and those of other investigators active in this area of study support our hypothesis that tumor-induced downregulation of SN immunity is the basis of the susceptibility of the SN to the survival and progression of the tumor cells that eventually generate clinically significant metastases.

Current efforts to reverse tumor-induced downregulation of nodal function may have some capacity to reduce the incidence of nodal metastases in melanoma patients, and by allowing the resumption of production of $CD8^+$Tcells, inhibit the survival and evolution of systemic metastases.

The system of primary melanoma/SNs provides a unique and highly relevant model for the study of the early stages of the metastatic process in melanoma.

ACKNOWLEDGMENTS

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35 Reversal of Immune Suppression in Sentinel Lymph Nodes

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CONTENTS

ABSTRACT

Minimally invasive intraoperative lymphatic mapping and sentinel node biopsy (LM/SNB) has become the standard approach for staging the regional lymph nodes for early-stage melanoma and breast cancer and has potential applications in other malignancies. The procedure requires close collaboration of surgeon, pathologist, and nuclear medicine physician. The strength of LM/SNB is its accuracy of detecting occult lymph node metastases while the therapeutic value of early dissection of occult metastases is yet unproved. RT-PCR analyses of either fresh frozen or paraffin-embedded sections of the sentinel lymph nodes may be more sensitive than hematoxylin and eosin (H&E) or immunohistochemistry but lack the specificity compared to conventional methods and limits in the availability of tissue specimens make RT-PCR impractical for routine use. LM/SNB allows for focused analysis of the matched primary malignancy and sentinel node. Research from human melanoma specimens suggest the sentinel

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node (SN) has molecular properties pointing toward immune dysfunction of these lymph nodes as compared with adjacent non-SNs and that reversal of this dysfunction may be an effective method of enhancing the immune response to melanoma.

Key Words: sentinel lymph nodes; immune dysfunction; granulocyte-macrophage colony stimulatory factor

1. INTRODUCTION

1.1. Historical Prospective

The controversy regarding the surgical management of the regional lymph nodes in early-stage melanoma began over 100 years ago. In 1892, Herbert Snow advocated wide excision and elective lymph node dissection (ELND) as a method to control the lymphatic permeation of metastases. His studies suggested a direct connection between the primary and the regional lymph nodes, indicating that treatment of melanoma should routinely include excision of these lymph nodes. Although multiple retrospective studies suggest a survival benefit for patients undergoing ELND in addition to excision of the primary melanoma, none of the randomized trials achieve a benefit for this procedure *(1)*. Each of these studies supports a potential survival advantage for early dissection of microscopically tumor-positive regional lymph nodes as compared to delayed (therapeutic) dissection of clinically apparent nodal metastases suggesting a benefit to patients to remove small volumes of tumor rather than waiting.

In 1992, Morton and associates described their initial experience with detection of occult regional lymph node metastases by intraoperative lymphatic mapping and sentinel node biopsy (LM/SNB) *(2)*. This technique, devised as an alternative to ELND, enables the surgeon to map the direct route of lymphatic spread from a primary melanoma to the regional drainage basin, and then selectively excise the first ("sentinel") draining lymph node(s) in this basin. Because the sentinel node (SN) is the most likely site of tumor cells in the regional lymph node basin, focused pathologic examination of the LM/SNB specimen is a useful method of ultrastaging the regional nodes. Complete lymph node dissection (CLND) is reserved for patients who are most likely to achieve a survival benefit from the procedure, i.e., those with metastasis to the SN. Patients without regional lymph node metastases could avoid the cost and morbidity of CLND.

Since the original seminal study, the accuracy of the LM/SNB has been improved with the addition of preoperative lymphoscintigraphy in all cases and intraoperative use of a handheld gamma probe to assist with identifying SNs *(3)*. The method of pathologic analysis has evolved to include relatively standard serial sectioning techniques and HMB-45 and Melan-A as immunostains *(4)*. The technique of LM/SNB has been shown by a number of investigators to be a reliable indicator of the tumor status of the regional lymph nodes, likely upstaging about 15% of cases compared to ELND. With over 15 years of experience, we and other groups have confirmed the low nodal recurrence rate and minimal morbidity from LM/SNB *(5)*. Based on these studies LM/SNB has replaced ELND and has become standard procedure for staging the regional lymph nodes.

While 2 major studies have evaluated the therapeutic value of LM/SNB both failing to confirm the therapeutic value of the procedure, this surgical technique has allowed for focused study of the physical interactions of the primary melanoma and matched sentinel (SN) and nonsentinel (non-SN) lymph nodes in a manner not previously done. Fresh and paraffin-embedded matched tissue can be compared in individual patients from residual material after routine analysis of the tissue which prevents competition between pathologist and molecular immunologist.

Because LM/SNB (and the primary melanoma) does not provide complete information of prognosis for these patients, we began to investigate if we could identify particular physical features that would assist with separating SN from non-SN but also provide additional information on the relative morphology and phenotype of these lymph nodes as a surrogate of disease progression.

2. ROLE OF THE IMMUNE SYSTEM IN SN

We observed that the presence of interdigitating cells (IDC), measured by immunohistochemistry (IHC) staining with antibody to S-100 protein, appear to be significantly less in number and density in SN versus matched non-SN. In addition, the majority of IDC in SN showed no dendritic cell processes. We also observed a significant reduction in the CD43 (T cell) expression, but no change in CD20 (B cell marker) expression from the lymphocytes in the paracortical areas of tumor-positive SN. *(6)*

While our observations of the physical appearance of SN and non-SN were novel, the significance of these findings is not known. Several investigators have noted, prior to the development of LM/SL, the relative lack of dendritic cells in regional lymph nodes (measured by IDC quantity) were significantly related to higher stage of malignancy and predicted subsequent recurrence for a variety of nonmelanoma malignancies (Table 1).

The concept of regional immunity being important for control of growth of malignancies is not new; our previous studies suggested that proximal (vs. more distal) lymph nodes had diminished ability to respond to lectins, interleukin (IL)-2), and had increased numbers of suppressor cells *(7–9)*. The control of primary tumor growth is regulated in part by the immune response from the regional lymph nodes *(10)*. Antigen presentation occurs with activation of $CD4^+$ and $CD8^+$ lymphocytes and induction of a variety of cytokines. Important for induction of this response is the activation of lymphoid-derived dendritic cells.

Dendritic cells were originally described based on their characteristic stellate appearance from the array of cell membrane processes *(15,16)*. These cells have been identified in almost all tissues, except the brain, and while few in number have been shown to be the most potent of antigen-presenting cells (APC). The importance of dendritic cell activation in cutaneous melanoma has not been well studied, especially in the context of the primary disease. Yet with increased understanding over the past 10 years of the importance of specific melanoma-related antigens to initiate an immune response through T-cell-derived mechanisms, the importance of activated dendritic cells cannot be understated *(17)*. The theoretical concept is that immature

Table 1
***(a)* Prognostic Significance of Dendritic Cell Infiltrates in Regional Lymph Nodes from Nonmelanoma Cancers**

Investigator (year)	*Tumor type*	*Implications*
Tsujitani (1987,1993) *(10,11)*	Gastric	Prolonged survival with ↑ dendritic cell
Zeid (1993) *(12)*	Nonsmall cell lung cancer	↑ survival with ↑ dendritic cell
Ambe (1989) *(13)*	Colorectal	Enhanced survival with ↑ dendritic infiltrates
Schroder (1988) *(14)*	Thyroid	↑ survival related to↑ dendritic cells in primary

Langerhans cells ($HLA\text{-}DR^+$, $CD80^-$, $CD83^-$, $CD86^-$) arising from the skin adjacent to the primary skin cancer, uptake protein antigens and migrate to the regional lymph nodes as mature APC ($HLA\text{-}DR^+$, $CD80^+$, $CD83^+$, $CD86^+$) *(18,19)*. Antigen uptake occurs early in the natural history of dendritic cells, and then mature, potent dendritic cells in the lymph nodes are then able to activate $CD4^+$ and $CD8^+$ T cells by presentation of peptide antigens in the context of the MHC restriction. Attempts to further augment the T cell responses in cancer patients, and particularly in melanoma, has had limited success *(20)*. Yet, with increased understanding of the role of dendritic cell activation both in animal models and *in vivo*, it has been shown that potent T cells can be induced from activated tumor-draining lymph nodes *(21,22)*.

The intended immune response to tumors has long focused on the specificity of the antigens and T cell responses measured by a variety of techniques (i.e., cytokine production, cytotoxicity of autologous tumor), yet it is clear that enhancement of those two components does not always lead to generation of immunity and tumor regression. Dendritic cells, as APCs, can initiate and modulate immune reactivity. The expression of costimulator markers B7.1 (CD80) and B7.2 (CD86) on mature dendritic cells can elicit a cytotoxic T lymphocyte (CTL) response *(23,24)*. While experiments with B7 molecules expressed on tumor cells alone were not successful for CTL production, these data suggest that the costimulatory molecules must be presented in the context of the dendritic cells for tumor rejection *(25)*.

The mechanisms by which mature dendritic cells activate resting T cells are becoming increasingly well understood. Dendritic cells have the essential molecules (CD80, CD86, CD40, etc.) on their surfaces that are critical for T cell activation acting through their cellular receptors (CD28 and CTLA4) in the context of antigen and MHC restriction.

A variety of approaches have been used for generation of specific T cell responses with dendritic cells, including pulsing dendritic cells with tumor lysates, peptides, or RNA from tumor cells or more recently with the creation of tumor cell–dendritic cell fusions. Each of these approaches is designed to increase T cell immunity with tumor antigens in concept with mature antigen-presenting dendritic cells *(26–28)*.

Another approach to increase immunity is through massive generation of mature antigen-presenting dendritic cells by coculturing immature cells with cytokines: granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, tumor necrosis factor (TNF-α), CD40L, Flt-3 ligand, or lipopolysacchride *(29–32)*. The greatest success in generating mature dendritic cells has been through the combination of coculturing techniques with GM-CSF and IL-4. IL-4 is generally not available and GM-CSF can produce large populations of mature dendritic cells alone.

There are also a number of agents known to be immunosuppressive to dendritic cells that may be important for immunologic tolerance and counterproductive to successful tumor rejection, including IL-10, tumor necrosis factor, IL-6, M-colony stimulating factor, TGF-β, and vascular endothelial growth factor *(33,34)*. Many of these factors are products of the primary tumor, adjacent stroma, and lymphocytes.

Even of further interest is the recent discovery of subset of dendritic cells that are thought to be immunosuppressive and/or toleragenic. There exists a subset of $CD_{123}{}^+/CCR_6{}^+$ dendritic cells that are known to express indoleamine 2, 3-dioxygenase (IDO) *(35)*. IDO is a rate-limiting enzyme in tryptophan catabolism *(36)* that metabolizes tryptophan into an intermediatary metabolite kynurenine, which is then catabolized to terminal metabolites of picolinic acid or quinolinic acid. Some of these metabolites have been shown to inhibit in vitro T cell proliferation. *(37)* In addition, IDO expressing dendritic cells have been shown to reduce T cell response in vivo. *(38)* Enhanced IDO activity has shown after stimulation with Th-1 type cytokines *(39)*, and can be induced from immature $CD_{123}{}^+/CCR_6{}^+$ dendritic cells by stimulation with interferon (INF)-α classic "proinflammatory" cytokine, with subsequent reduction in T cell proliferation *(35)*. Furthermore,

whereas stimulation of mature dendritic cells (DCs) with INF-γ resulted in downregulation of IDO activity, INF stimulation of DCs that are matured in presence of IL-10 resulted in enhancement of IDO. IDO's relevance in melanoma local immune downregulation has suggested by the finding that IDO^+ DCs are present in melanoma SNs.

On the other hand, recombinant human GM-CSF and Flt-3 ligand, along with IL-4, maybe able to reverse these features of a dendritic cell dysfunction *(40–43)*. The rationale for examining lymph node populations over blood pools, is that the SN may be more reflective of the true interaction between the primary skin cancer and the local/regional environment. GM-CSF as an agent to reverse the phenotypic appearance noted in SN. The concept that lymph node phenotype can be reversed is unique and possibly may represent a novel approach to offering therapy directed at the regional lymph nodes early in the natural history of melanoma when chances of cure are much higher than more advanced stages.

3. THE ARGUMENT FOR FOCUSED ANALYSIS OF SN

Our initial experience with LM/SL for early-stage melanoma demonstrates that the SN could be differentiated from non-SN based on the presence of blue dye staining or through the use of a handheld gamma probe to detect radioactivity in the SN *(44)*. The procedure is performed in three steps: (1) Preoperative cutaneous lymphoscintigraphy performed on the day of surgery (or at most 24 h prior) with $\leq$500 μCi of technetium 99m sulfur colloid injected intradermally at the primary site and imaged with a gamma-particle-sensitive camera to allow nuclear medicine physicians to mark the site of concentrated radiation in the draining lymph node basin. (2) During surgery ~1 cc of isosulfan blue (*Lymphozurin*, Tyco International, Exeter, NH) is injected intradermally at the primary melanoma site and a small incision is made at the lymph node site marked by the nuclear medicine physician. A blue-stained and radioactive SN is removed and marked for the pathologist. Sentinel lymph nodes are defined as blue stained or have greater than 10:1 ratio in radioactivity as compared to other adjacent lymph nodes. Lymph nodes that are neither blue stained nor radioactive are termed non-SN. (3) Pathologic analysis of SN and non-SN includes ~10 serial sections of the bivalved nodes with use of hematoxylin and eosin (H&E) staining along with separate sections examined with IHC staining using melanoma-sensitive antibodies to MART-1, HMB-45, and S-100 proteins. Pathologic analysis is performed on permanent section specimens alone. Completion lymph node dissection is performed only in cases with tumor-positive SN.

We have performed LM/SL in over 2,000 patients over the past 15 years (average 130/year). The number continues to increase as the procedure becomes more accepted as standard of care. The success of identifying the SN in each basin approaches 98% and has been validated through a US NCI-supported trial (P01 CA29605, Donald L. Morton, MD, Principle Investigator) headquartered at our Institute *(45)*. Tumor-positive SN are found in ~20% of cases, 75% of cases with single tumor-positive node. Approximately 50% of patients with tumor-positive dissections will recur and most of these patients will die of their disease. Long-term follow-up (median = 51 months) suggests 16–18% of patients with tumor-negative dissections will ultimately recur; ~2% first in the dissected basin and 14–16% at distant sites only. More extensive pathologic analysis of the tumor-negative SN rarely leads to identification of the missed metastasis.

More recent surgical focus has been on methods to determine if more than just the SN has metastases. Patients with disease confined to the SN alone could be spared the cost and morbidity of complete lymph node dissection. We examined clinicopathologic features from 218 patients with tumor-positive SN to determine factors to predict if non-SN contained metastases. Only

Table 2
Factors Predictive of More Than One Tumor-Positive Lymph Node Following LM/SL

Factor	*RR [95% CI]*	*p value*
Primary tumor thickness >3 mm	2.96 [1.47, 5.99]	0.002
SN metastasis ≥2 mm in size	2.93 [1.41, 6.06]	0.003
# of +SN ≥2	2.05 [0.98, 4.27]	0.054
Patient age >60	1.84 [0.92, 3.68]	0.082
High mitotic rate of primary	1.92 [0.87, 4.20]	0.1

primary tumor thickness and tumor burden were predictive although neither parameter was accurate for individual cases (Table 2). The significance of this large clinical experience is that it provides a meaningful database of clinical information and pathologic specimens.

4. IMMUNOHISTOCHEMISTRY ANALYSIS OF SENTINEL AND NON-SENTINEL LYMPH NODES FOR DENDRITIC CELL MARKERS

S-100 IHC staining intensity (as a measure of IDC area and density) for matched SN and non-SN specimen was performed using an IBM PC-assisted image analysis system (planar morphometry, version 2.1, Southern Micro Instruments, Atlanta, GA). We obtained lymph node tissue blocks from 21 matched pairs of SN and non-SN. The 4-μm-thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks along the long axis at the periphery of the lymph nodes. The polyclonal antibody S-100 protein was used to identify IDC and monoclonal antibodies CD43 and CD20 (Dako Corp., Carpinteria, CA) used to identify T cells and B cells, respectively. Paraffin sections were examined with the assistance of an image analyzer. Relative IDC area (SN: $2.0 \pm 0.68\%$ vs. non-SN: $23.5 \pm 1.4\%$, $p = 0.0001$, two-sided t tests with equal variance) and density (SN: $14.5 \pm 2.8/\text{mm}^2$ vs. non-SN: $114.3 \pm 6.6/\text{mm}^2$, $p < 0.001$) were significantly diminished in SN as compared to non-SN. The presence or absence of tumor in the SN and non-SN did not appear to influence these results, although only four pairs of lymph nodes had metastases in the SN. Similarly, a significantly ($p = 0.0001$) higher proportion of SN ($66.02 \pm 4.73\%$) lacked dendrites than non-SN ($36.99 \pm 1.18\%$) suggesting these DC were not functional. The relative area of $CD20^+$ (B cells) stained cells were no different (SN: $24.0 \pm 2.1\%$ vs. non-SN: $28.5 \pm 1.4\%$, $p = 0.14$) between SN and non-SN, but CD43 (T cell) expression was significantly ($p < 0.0001$) lower in SN. The implication from our results is that the physical appearance of IDC populations of SN and non-SN were significantly different.

To further assess the significance of these changes, we analyzed matched SN and non-SN from another 20 patients undergoing LM/SL. IHC staining was performed by using 4-μm-thick fresh frozen sections from peripheral tissue from SN and non-SN. Monoclonal antibodies to CD40, CD80, CD86, CD28, and CD152 (CTLA-4) were obtained from Pharmigen (San Diego, CA). CD80 (B7.1) and CD86 (B7.2) are costimulatory molecules known to be important for activating T cells *(46)*. Both are present primarily on activated (mature) dendritic cells. CD40 expression is also seen as an early marker of mature dendritic cell. The corresponding T cell receptors (CD28 and CTLA-4) are necessary for dendritic cell presentation and activation of T cells *(47)*. In our experiments, CD40 expression (IHC staining) was significantly reduced in SN as compared to non-SN. CD80, CD86, CTLA-4, and CD28 were only minimally expressed

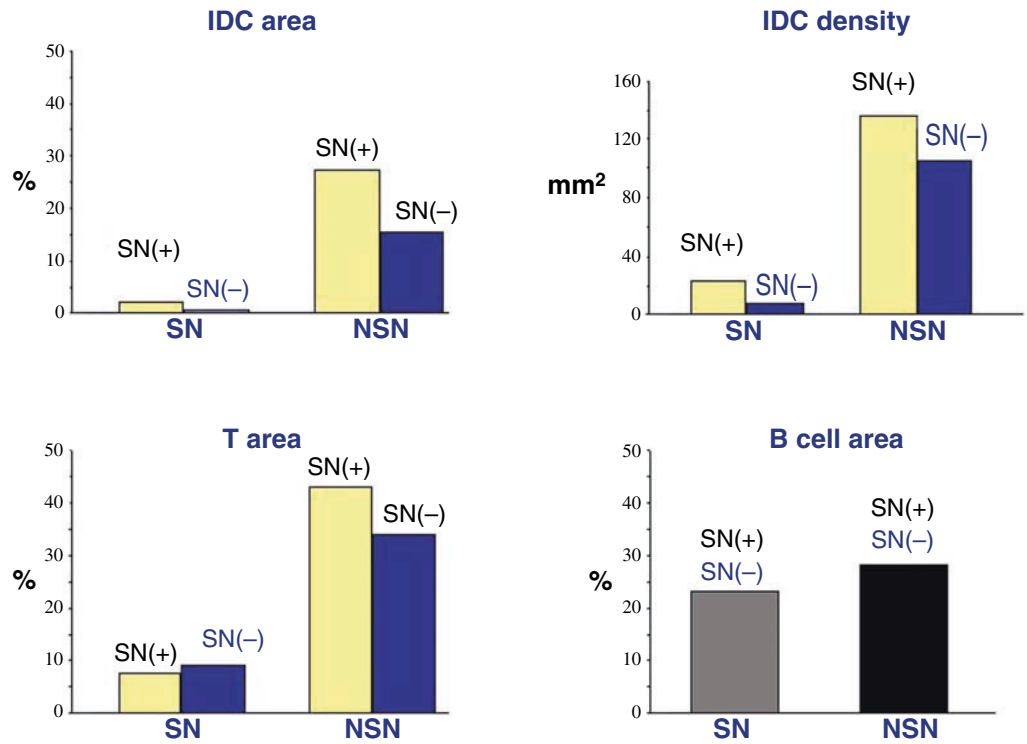

Fig. 1. IHC staining with antibody to S-100 demonstrates significant reduction in IDC area and density in SN versus non-SN (NSN). T cell (CD43) area was also reduced in SN compared to non-SN while B cell (CD20) area was not altered. The presence (SN+) or absence (SN–) of metastases to the SN did not appear to alter the differences in IDC area or density. (*see* Color Plate 22)

from each SN and non-SN match. The low level of expression noted for these markers may be related to the sparse distribution of dendritic cells and the sensitivity of the antibodies used to evaluate the lymph nodes (Fig. 1 and Color Plate 22).

5. GENE EXPRESSION OF DENDRITIC CELL MARKERS OF ACTIVATION FROM SENTINEL AND NON-SENTINEL LYMPH NODES

Because of the sparse distribution of dendritic cells in SN and non-SN, and the low level of marker expression detected by IHC staining, we evaluated gene expression of CD80, CD86, CD40, CD28, and CTLA-4 by semiquantitative RT-PCR.

Twenty-four patients underwent LM/SL, 20 (83%) patients had matched SN and non-SN. A total of 26 matched lymph node sets were evaluated. In three cases, non-SN were not identified at surgery, and in two cases insufficient mRNA was present from the lymph nodes (if nodes were <1 cm in size, fresh RNA was not obtained). Lymph nodes were immediately processed by freezing and tangential sections cut ~4-μm-thick from a side of the node parallel to the longest axis of the specimen. In brief, RNA was isolated from lymph node extracts using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) and reverse transcribed to coda. To assess the quantity of mRNA, each of the markers were evaluated separately and amplified using published primer sequences. The housekeeping gene GAPDH was used as a control for comparison, and

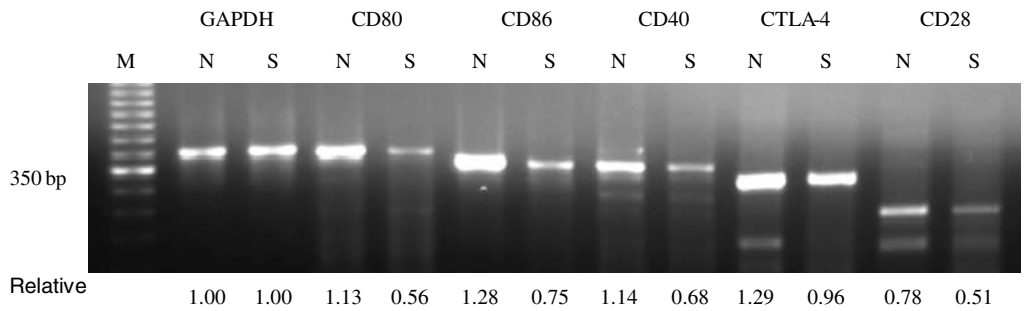

Fig. 2. Representative image demonstrating relative diminished expression of dendritic markers for SN (S) as compared to non-SN (N) matches. GAPDH serves as standard control for each specimen.

CD2 and CD20 also served as quantitative B cell and T cell controls. We compared relative gene expression for our markers by analyzing band intensity by laser densitometry readings, correcting for GAPDH expression from each specimen (Fig. 2).

In a majority of cases, relative inflammatory cell marker expression was lower in SN versus non-SN: CD80 (77%), CD40 (85%), CTLA-4 (88%), and CD28 (85%). The mean (and standard deviation) level of dendritic marker expression (relative to GAPDH) was significantly ($p < 0.05$) lower for SN as compared to non-SN (based on the 26 matches of SN and non-SN). Gene expression CD2 and CD20 were essentially no different. The significance of these findings was the creation of the methodology and standard operating procedures for analyzing SN and non-SN matches by semiquantitative RT-PCR. Any conclusions drawn from semiquantitative techniques for assessing quantitative gene expression must be considered in the context of the experiments performed (Fig. 3).

We concluded that the gene expression suggestive of dendritic cell maturity was diminished in SN versus non-SN unrelated to the expression of the T (CD2) and B (CD20) cell markers. (We did not examine CD43 gene expression.) We reviewed our data to determine if these results could be related to the initial biopsy reaction or injury to the SN from the radiopharmaceuticals injected at the primary site used for imaging the SN for LM/SL. All cases of LM/SL were performed at least 14 days and up to 60 days after initial skin biopsy. There appeared to be no relationship of time lapse from biopsy to LM/SL that would explain our results. The amount of radioactivity that reaches the SN from the lymphoscintigraphy is approximately 1% of the injected radiopharmaceutical. The dose of radiation was determined by the relative exposure time to the tissue. We found no relationship between dose and time from lymphoscintigraphy to surgery to explain our results. The dose of technetium used should not affect dendritic cell viability *(48)*.

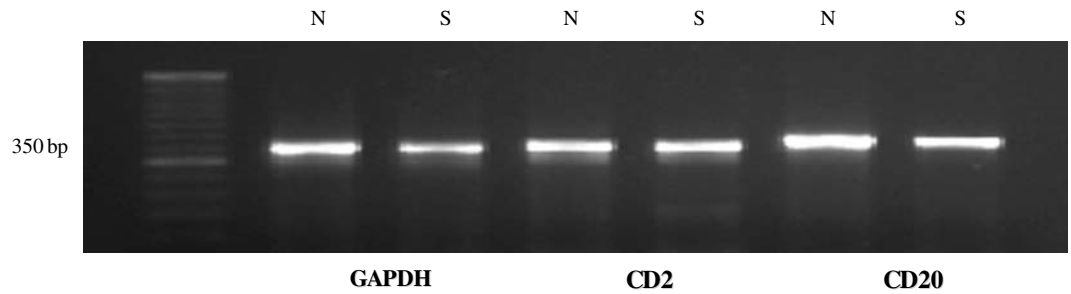

Fig. 3. Representative experiment demonstrating consistent expression of CD2 and CD20 gene expression from SN (S) and non-SN (N).

6. EVALUATE GENE EXPRESSION OF TH-1- AND TH-2-RELATED CYTOKINES FROM SENTINEL AND NONSENTINEL LYMPH NODE MATCHES

The process of T cell activation is related to the expression of Th-1 and Th-2 cytokines that regulate $CD4^+$ and $CD8^+$ lymphocyte function. We examined cytokine gene expression from 29 matched SN and non-SN pairs from 24 patients (mRNA was obtained as previously described) and processed by semiquantitative RT-PCR using specific primers to the Th-1 cytokines: IL-2 and IFN-γ and Th-2 cytokines: IL-4 and IL-10. We found only minimal expression of IL-2 or IFN-γ expression in any of the samples. The immunosuppressive cytokine IL-10 was expressed at a higher level (SN to non-SN ratio >1) in 80% of SN and non-SN matches, and relative IL-10 expression was greater for SN than non-SN. The presence of metastases in five SNs had no effect on our findings, likely related to the small sample size (5 vs. 24).

We evaluated IL-10 expression in the context of known prognostic factors for early-stage melanoma, including gender, age, primary site and thickness, and lymph node status. Univariate

Table 3
Univariate and Multivariate Analysis of Semiquantitative RT-PCR Expression of IL-10 from SN and non-SN Pairs

	p values	
Factor	*Univariate*	*Multivariate*
Age (>50 years vs. ≤50 years)	0.02	0.05
Gender (women)	0.02	0.10
Primary site (extremity vs non-extremity)	0.13	0.52
Primary thickness (>1.5 mm)	0.01	0.048
SN status	0.28	0.4

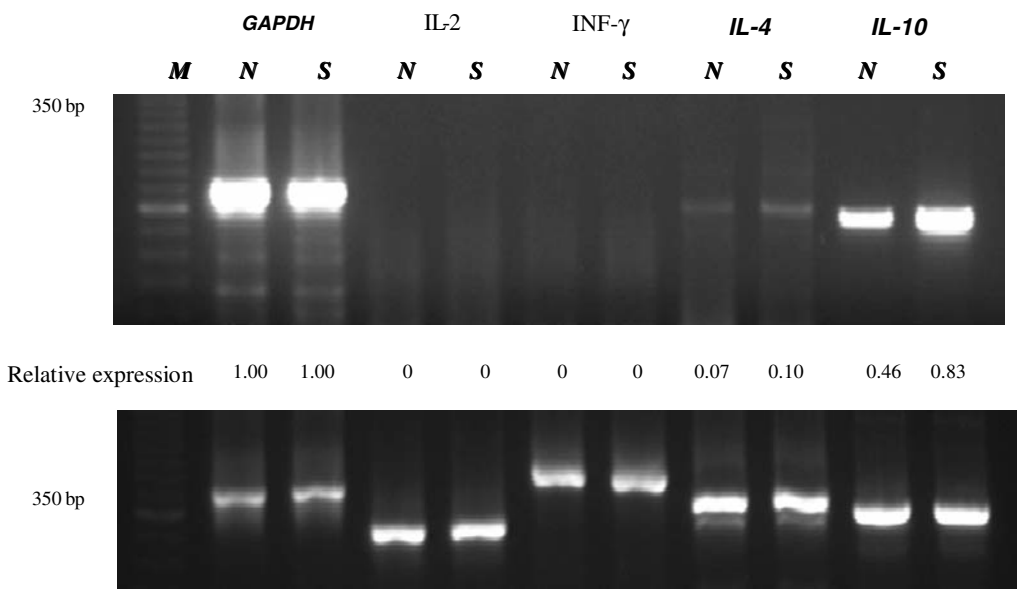

Fig. 4. Semiquantitative RT-PCR analysis of representative experiment demonstrating significantly higher expression of IL-10 from the SN (S) than non-SN (N) from a single patient. We detected only minimal expression of IL-2, IFN-γ, and IL-4 from S or N specimens. Bottom gel represents sample controls for each cytokine.

and multivariate statistical analysis demonstrated IL-10 gene expression was significantly higher for older (>50 years) patients and thicker (>1.5 mm) primaries (Table 3). Gender, primary site, and SN tumor status did not correlate with IL-10 levels, but the sample sample size may have prevented further correlations. CD2 (T cell marker) was no different from SN and non-SN specimens, but CD20 (B cell marker) marker expression was significantly ($p = 0.01$) higher from patients with tumor-positive than negative SN (data not shown) (Fig. 4).

7. DEVELOPMENT OF REAL-TIME QUANTITATIVE RT-PCR ANALYSIS FOR EVALUATION OF SENTINEL AND NONSENTINEL LYMPH NODE MATCHES

Precise quantification of gene copy number using semiquantitative RT-PCR has traditionally been difficult to interpret and is reflected in our own results. There are several criticisms of commonly used semiquantitative PCR techniques which include labor-intensive endpoint dilutions which can produce a wide range of values and postamplification handling of PCR product which is prone to contamination *(49–53)*. Real-time quantitative RT-PCR is a relatively new technique that provides very accurate and reproducible quantification of DNA or RNA copy numbers by using a dual-labeled fluorogenic probe conjugated with a reporter fluorescent dye at the 5′ end and a quencher dye at the 3′ end *(54)*. As the target sequence is amplified by the primers, the probe anneals and is cleaved by the 5′ nuclease activity of the Taq DNA polymerase. This causes separation of the reporter and the quencher dye which generates a fluorescent signal. Each cycle of PCR amplification cleaves additional reporter molecules, increasing the intensity of the signal in proportion to the amount of transcript produced, and is detected by the real time system as the PCR product accumulates. Quantitation of the PCR product is performed in the following manner. A gene of interest is chosen and mRNA sequence is identified. A forward/reverse primer set and probe are subsequently designed and the mRNA segment is then amplified using PCR. Subsequently, the amplicon is separated using agarose gel electrophresis and recovered. The amplicon is then cloned into a carrier plasmid and transfected into a cell line for plasmid amplification, which is subsequently isolated and purified. The concentration of plasmid is determined by optical densitometry, then serially diluted to generate a standard curve. The sample in question is then evaluated using quantitative RT-PCR and the resultant fluorescence is measured against the standard curve to determine mRNA copy number of the gene of interest, in that particular sample. An example of a standard curve generated for GAPDH is shown in (Fig. 5 and Color Plate 23).

This technique is faster, more accurate and less labor-intensive than older techniques, and does not require handling of the PCR product post amplification, thus avoiding carry over contamination. In addition, real-time PCR allows multiple quantification in a single tube, which improves accuracy and speed while reducing costs *(49,51,55)*.

We have performed real-time quantitative PCR using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) and Taqman assay to determine cytokine gene expression from matched SN and non-SN from ten patients. Standard curves were prepared for IL-10 and the endogenous reference, β actin. The relative quantity of IL-10 and β actin for each sample was determined from the standard curves, and the normalized value of cytokine expression was calculated by dividing by the β actin value. Reproducible levels of cytokine expression were found in all ten patients upon repeat assay and real-time quantitative PCR values were compared with the relative results of semiquantitative PCR assays. In all of the ten cases, values were concordant, but real-time RT-PCR allowed us to evaluate for cycle number differences. IHC staining (R&D Systems, Minneapolis, MN) was used to verify results for IL-10 expression in nodal tissue As mentioned previously, SN display an immune down-regulatory morphology

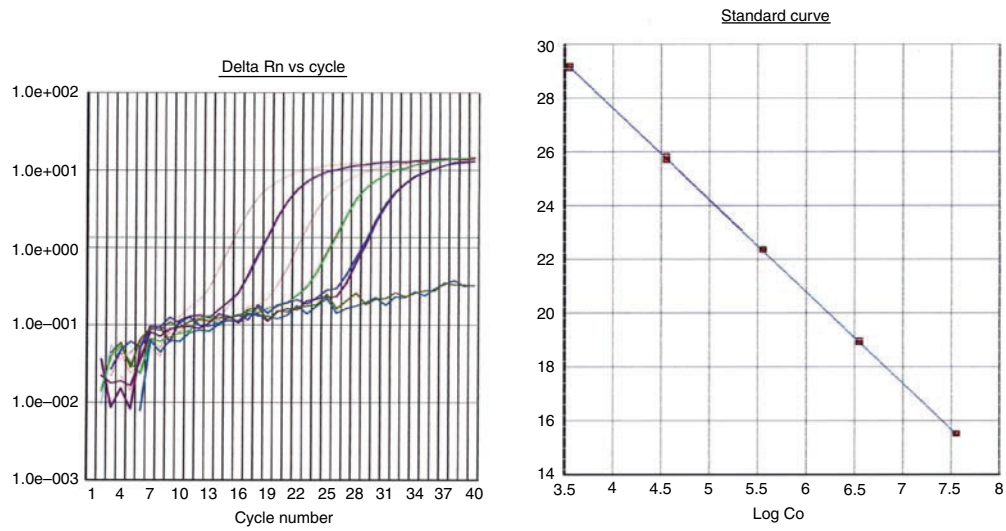

Fig. 5. Sample amplification plot for GAPDH standards demonstrating the threshold cycle (C_t) for each of the serial dilutions. In the second graph, the C_t's are plotted against the Log(quantity—C0), providing a standard line with a slope and quality of fit. (*see* Color Plate 23)

when compared to the non-SN. This feature of SN morphology, however, was found to be present even in absence of SN metastasis, which suggests that tumor–SN immune interaction precedes the SN metastasis.

Using quantitative RT-PCR, we examined differential immune regulatory gene expression in 21 matched pairs of SN and non-SN. All of the 21 patients underwent wide excision and LM/SL for a biopsy proven melanoma. At surgery, a pair of SN and non-SN were harvested (see earlier definition of SN and non-SN) and immediately preserved in a vial containing RNAlater (Qiagen Inc., Valencia, CA) for RNA preservation. Out of 21 patients, 13 (62%) had postbiopsy residual tumor (either in the SN or primary site) at the time of wide excision (based upon routine pathology analysis), and 8 (38%) showed no evidence of any postbiopsy residual tumor. The relative levels of immune regulatory gene expression in the SN (compared to matching non-SN) are shown in presence or absence of neighboring residual tumor (Fig. 6 and Color Plate 24). The presence of residual tumor is associated with up regulation of immune regulatory gene expression in the SN. As mentioned earlier, the dendritic cells expressing IDO can induce immune downregulation by inhibiting T cell proliferation and induction of toleragenic T cells *(56,57)*. The data show that IDO gene expression is upregulated in the SN (compared to non-SN) in presence of residual tumor, suggesting tumor associated, and possibly tumor induced, immune downregulation in the SN.

Working with fresh lymph node tissue, despite its obvious and critical advantage for evaluating in vivo gene expression, has its inherent limitations related to limited quantity of specimen available for analysis. Furthermore, several years of follow-up is required to assess its functional significance pertaining to prognosis and ultimate outcome. The ability to perform similar experiments using formalin-fixed, paraffin-embedded tissues can greatly circumvent these restrictions. The usage of stored archival tissues solves the problem of limited tissue availability and lack of long-term follow-up. The concept of utilizing archival tissue for RNA extraction and subsequent reverse transcription and PCR amplification has been available for the past decade. Several investigators have successfully utilized this method for PCR amplication of genes for actin *(58)*, thymidine kinase, ribosomal RNA, tyrosinase, and Melan-A/MART-1 *(59,60)*. However, their methods were limited to semiquantitative PCR, with no documented RNA

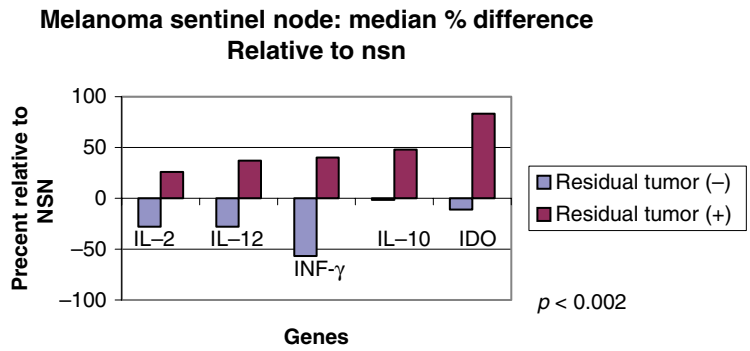

Fig. 6. Significant difference in cytokine gene expression in the SN compared to the NSN (non-SN). The relative patterns of cytokine expression are reversed in the presence or in absence of residual melanoma found during LM/SL. Presence of residual tumor significantly increases the levels of cytokine expression in the SN. Most dramatic increase is noted in the expression of IDO, which is an enzyme expressed in immune downregulatory dendritic cells *(56,57)*. (*see* Color Plate 24)

quality or duration between tissue fixation and RNA extraction. We have evaluated various extraction methods and subsequently modified and optimized the technique for real-time quantitative PCR. Briefly, the paraffin blocks are cut in *20*-μm sections—two to four sections are used. The paraffin sections are deparaffinized using xylene–ethanol wash, followed by Proteinase-K

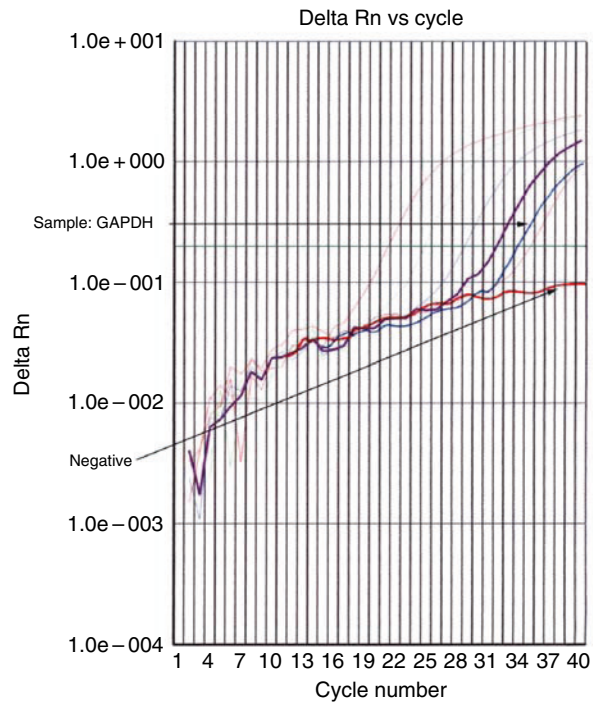

Fig. 7. Real-time quantitative PCR amplification plot for GAPDH, a standard housekeeping gene is shown. The RNA was extracted from an archival tissue that was fixed in formalin and embedded in paraffin. The figure demonstrates successful quantitative measurement of GAPDH gene expression in such tissue sample. (*see* Color Plate 25)

digestion. The sections are then homogenized and vortexed in presence of Tri-Reagent (MRC, Cincinnati, OH). Following centrification, the aqueous phase is transferred to BCP (MRC, Cincinnati, OH), vortexed, then centrifuged again. The aqueous phase is separated and precipitated in iso-propanol at –20°C. After three cycles of centifuge–EtOH wash, the RNA pellet is dried and dissolved in nuclease-free H_2O. The concentration and initial RNA quality is determined by optical densitometry, which usually results in RNA quality of *1.66*–1.74 on A260/A280 spectrometry reading. Subsequently, the final RNA quality check is done using the Agilent Bio-Analyzer (Agilent Technologies, Wilmington, DE). The real-time quantitative PCR amplification plot for GAPDH using RNA sample extracted from formalin-fixed and paraffin-embedded tonsil tissue is shown in (Fig. 7 and Color Plate 25) *(56,60–65)*.

8. EVALUATION OF GM-CSF TO REVERSE THE PHENOTYPIC CHANGES TO SENTINEL LYMPH NODES

We elected to evaluate GM-CSF as a cytokine to "reverse" the morphologic changes observed in SN versus non-SN, based on its properties to mature dendritic cells. While most studies using GM-CSF as therapy for melanoma were based on repeated subcutaneous inoculations for up to ~21 consecutive days, we sought to examine the utility of GM-CSF delivered intradermally around the primary melanoma *(57,66)*. Our rationale for intradermal injection was based on the premise that a majority of the drug would be delivered through the lymphatics to the SN.

Fifteen consecutive patients received a single preoerative intradermal injection of GM-CSF (*Sargramostim*, Berlex Corp., Seattle, WA, gift of Mark Gilbert, MD) 2–5 days prior to LM/SL. The first five patients received GM-CSF at 100 μg/m^2, a second group of 5 receiving 150 μg/m^2, the last five patients receiving 200 μg/m^2. LM/SL was performed as previously described. Thirty-four SN and 9 non-SN were resected from the 15 patients. SN and non-SN matches (and untreated SN) were stained by IHC to S-100 and evaluated using the IBM image analyzer.

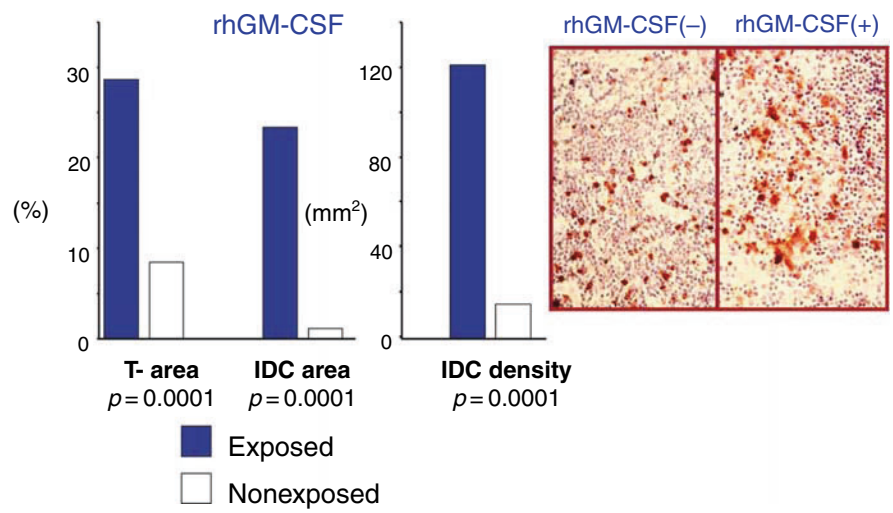

Fig. 8. Comparison of morphologic features of 34 GM-CSF-exposed SN to 21 SN from patients who had not received peritumoral GM-CSF. Data are based on morphometric analysis with the observer blinded to the source of the SN. The relative T cell area, IDC area, and density were significantly higher for SN in patients who had received preoperative GM-CSF.

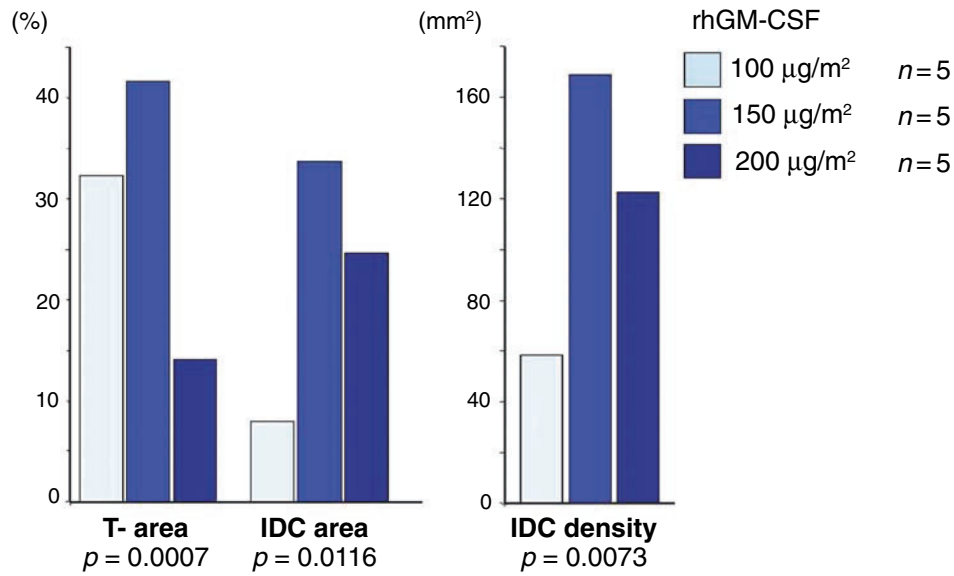

Fig. 9. Relative T cell and B cell area and IDC density in SN based on preoperative dose of GM-CSF. Patients receiving GM-CSF 150 µg/m^2 appeared to have the greatest morphologic change to the SN. (*see* Color Plate 26)

Our experience with a single peritumoral (primary site) injection of GM-CSF 2–5 days prior to LM/SL suggests that the morphologic features (IDC area and density, based on S-100 staining) observed from treated SN were significantly higher than from untreated SN (Fig. 8).

The nine non-SN resected from GM-CSF-treated patients were compared to 21 non-SN from patients not receiving the single peritumoral injection of GM-CSF. Lymph nodes were evaluated for relative T cell area, IDC area, and density. The relative T cell area, IDC area, and density were no different between GM-CSF-exposed and nonexposed non-SN (not shown).

The data were reexamined based on the initial preoperative dose of GMCSF. The relative T cell area and IDC area and density were greatest in the SN from the five patients who received the intermediate dose of 150 µg/m^{2}of GM-CSF (Fig. 9 and Color Plate 26). We examined patient- and tumor-related factors from each of the 15 patients treated with the various doses of GM-CSF. We did not detect any differences in patient or primary tumor characteristics to explain the results observed with the various doses (data not shown).

9. SIGNIFICANCE OF GM-CSF DATA

The finding of GM-CSF reversing the immunoprofile of SN and non-SN is significant and suggestive that the lymph nodes can be altered and may be a target for immunotherapy.

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36 Vaccine-Primed Lymph Node Cells in the Adoptive Immunotherapy of Cancer: Presence of Host Immune Suppression Induced by Established Cancer

Shuang Wei, PhD, Andrew B. Shreiner, BS, and Alfred E. Chang, MD

Contents

ABSTRACT

Lymph nodes represent critical lymphoid compartments where antigen-presenting cells interact with T cells to propagate cellular immune responses. As such, they represent a unique source of "pre-effector" T cells that can be induced by the regional inoculation of tumor antigen as a vaccine. Utilizing this concept, we have been able to generate tumor-reactive T cells from vaccine-primed lymph nodes (VPLNs) that can mediate tumor regression in adoptive immunotherapy. This chapter describes how this pre-effector response in VPLNs can be suppressed by the presence of established systemic tumor. This suppression involves the B7-H1/PD-1 axis as well as TGF-β. Methods to block these suppressive mechanisms will be important in improving future adoptive cellular therapy approaches.

Key Words: T cells; immune suppression; adoptive immunotherapy; vaccines; lymph nodes

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1. INTRODUCTION

Adoptive T cell immunotherapy is defined as the passive transfer of tumor-reactive T cells into the tumor-bearing host that results in the direct or indirect destruction of established tumors. Utilizing inbred strains of mice where transplantable tumors were established, investigators in the 1950s and 1960s were able to cause regression of established tumors by adoptively transferring lymphocytes derived from normal mice that were immunized with various tumor vaccine preparations. This provided the proof-of-principle that the transfer of appropriately immunocompetent cells can mediate tumor regression upon transfer to a syngeneic host. The major obstacles to developing these therapies clinically was the ability to expand ex vivo sufficient quantities of T cells, and more importantly, being able to isolate tumor-reactive T cells from the tumor-bearing host.

Rosenberg and colleagues have pioneered the use of tumor-infiltrating lymphocytes (TILs) in the treatment of patients with advanced melanoma. TIL cells have been characterized to have specificity in recognizing antigens expressed by the tumor from which they have been derived. In this chapter, we will review another potential source of lymphoid cells that are tumor reactive and can be used in adoptive immunotherapy.

2. TUMOR-DRAINING LYMPH NODE CELLS: ANIMAL STUDIES

An alternative source of effector T cells for adoptive immunotherapy are from lymph nodes. Lymph nodes are important secondary lymphoid organs where dendritic cells (DCs) interact with T cells to initiate a primary immune response *(1)*. Our laboratory has characterized the effectiveness of either tumor-draining lymph node (TDLN) or vaccine-primed lymph node (VPLN) cells as effector cells in adoptive immunotherapy *(2,3)*. The generation of effector T cells from TDLNs or VPLNs is restricted by the immunogenicity of the tumor cells, kinetics of response within the draining lymph nodes and requires secondary activation ex vivo for differentiation and expansion of the lymphoid cells to become immunocompetent effector cells in adoptive immunotherapy.

Utilizing the poorly immunogenic B16-BL6 melanoma cell line, we have demonstrated in animal models the ability to generate effector T cells from TDLN that were capable of mediating the regression of both experimentally induced and spontaneous metastases *(4)*. In those studies, the growth of B16-BL6 tumor cells inoculated intradermally did not elicit effector cells in the TDLN. It required the coadministration of an immune adjuvant, in this case, *Corynebacterium parvum*, to induce effector cells in the TDLN that could be activated secondarily by anti-CD3 monoclonal antibody and expanded in IL-2.

We have also examined alternative vaccine strategies to prime draining lymph nodes as a means of eliciting effector cells for adoptive immunotherapy. In a preclinical model utilizing the B16-BL6 melanoma, we examined the utility of genetically modifying tumor cells with different cytokine genes as a means of generating vaccines that could prime draining lymph nodes *(5)*. We found that granulocyte-macrophage colony-stimulating factor (GM-CSF) was superior to other cytokines in enhancing the immunogenicity of the melanoma tumor cell line. In an adoptive immunotherapy model, we demonstrated that B16-BL6 tumor cells transduced to secrete GM-CSF were superior than tumor cells admixed with a bacterial adjuvant in eliciting effector cells in VPLN *(6)*. Based on these observations, we conducted a pilot study in patients with stage IV melanoma where autologous tumor cells were transduced retrovirally to secrete GM-CSF and were utilized as vaccines to generate VPLN cells for adoptive immunotherapy *(7)*. In that study, we found that the local release of GM-CSF at the vaccine site resulted in a significant infiltration of DC into the microenvironment. One patient out of five went on to have a complete clinical response to therapy that has been durable.

Our laboratory has continued to optimize the culture conditions by which TDLN or VPLN cells can be activated and expanded ex vivo. It was apparent that effector cells that release a type 1 cytokine profile in response to tumor antigen were more capable of mediating tumor regression compared to effector cells that release a type 2 cytokine response *(8,9)*. Furthermore, we have found that only a small subset of cells within primed lymph nodes constitute the "pre-effector" cell population that mediate the antitumor response. These are $CD8^+$ and $CD4^+$ cells that express P-selectin ligand^{high} markers and released the greatest amount of IFN-γ in response to tumor antigen *(10)*. The cultured Plig^{high} TDLN were 10- to 20-fold more active against established pulmonary micrometastases than cultured unfractionated TDLN, and >30-fold more active than cultured TDLN cells depleted of the Plig^{high} fraction before expansion.

Besides utilizing anti-CD3 mAb to secondarily activate tumor-primed lymph node cells, we have investigated the addition of costimulatory signals to generate effector cells for adoptive immunotherapy. This has included the use of anti-CD28 mAb in addition to anti-CD3 to activate lymphoid cells. In preclinical animal studies, this has resulted in enhanced type 1 and 2 cytokine responses of activated tumor-primed lymphoid cells in response to tumor antigen *(11)*. $CD4^+$ cells are generated via this method which was not the case with anti-CD3 activation alone. TDLN cells activated with anti-CD3 and anti-CD28 were therapeutically more effective than anti-CD3 activated cells in adoptive immunotherapy. We are currently conducting a clinical trial utilizing this method of activating VPLN cells for adoptive therapy. In other studies, we have also evaluated the effect of anti-4-1BB mAb in conjunction with anti-CD3/anti-CD28 mAb to activate tumor-primed lymph node cells. The addition of anti-4-1BB resulted in TDLN cells that polarized the cytokine release response of the activated cells to a type 1 response which correlated with enhanced antitumor responses in adoptive immunotherapy *(12)*. The enhanced in vivo efficacy of cells activated with anti-4-1BB may be related to improved survival and proliferation of the activated cells after adoptive transfer *(13)*.

3. VACCINE-PRIMED LYMPH NODE CELLS: CLINICAL STUDIES

These observations resulted in an early pilot study we conducted in patients with advanced melanoma and renal cell cancer who were vaccinated with irradiated autologous tumor cells admixed with the bacterial adjuvant, *Bacillus Calmette Guerin (BCG)*, who went on to receive VPLN cells that were activated with anti-CD3/IL-2 *(14)*. In that study, we were able to demonstrate the preferential expansion of $CD8^+$ T cells from the VPLN that responded to autologous tumor cells in vitro with MHC class I restricted secretion of IFN-γ and GM-CSF cytokines. In addition, clinical responses were noted in patients with either melanoma or renal cell cancer. A subsequent phase II trial of anti-CD3 activated VPLN cells was performed in patients with stage IV renal cell cancer *(15)*. Durable tumor responses were noted in 27% of the treated patients which correlated with the cytokine profile released by the VPLN cells in response to autologous tumor cells. With a greater IFN-γ:IL-10 ratio, the more likelihood a response was observed (Fig. 1). We are currently conducting a follow-up trial in patients with stage IV renal cell carcinoma (RCC) where individuals receive lymphodepleting chemotherapy prior to the infusion of VPLN cells that are activated with anti-CD3 and anti-CD28 monoclonal antibodies. IL-2 is also administered concomitantly with the cell infusion.

Our clinical efforts have been based on our animal models where non-tumor-bearing hosts are used as donors of tumor-primed lymph node cells. However, this is not the situation we are faced with clinically. In the latter situation, tumor-primed lymphoid cells have to be harvested from the tumor-bearing patient. It is clear from various laboratories, that the tumor-bearing state results

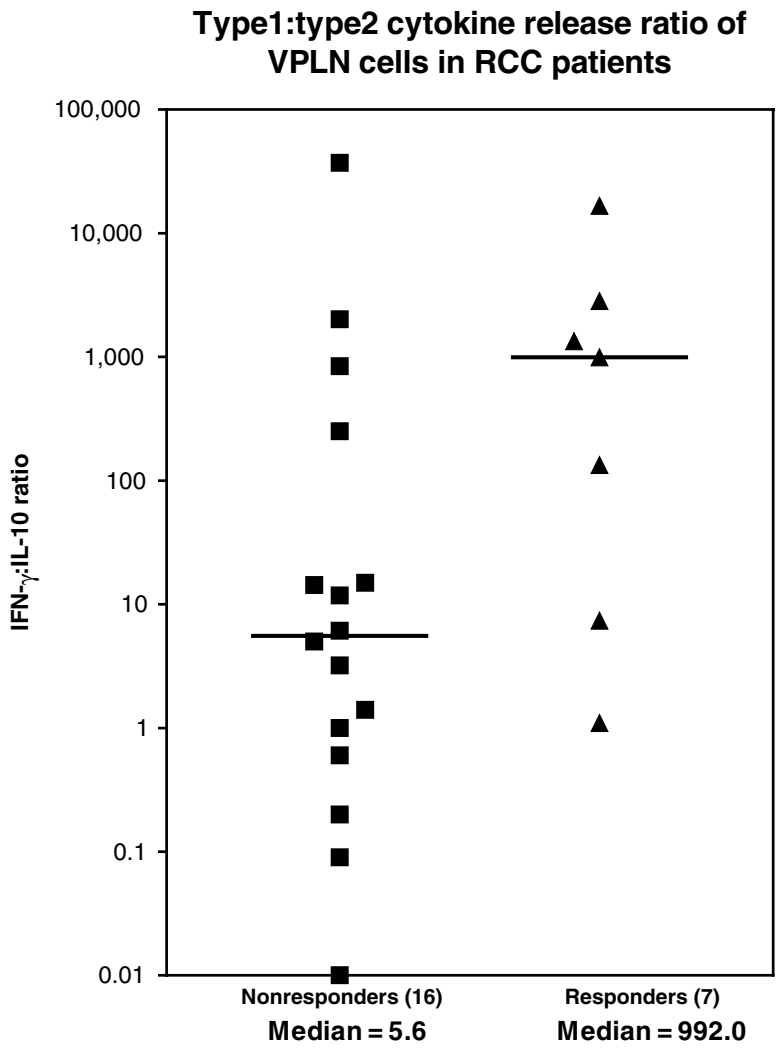

Fig. 1. Correlation of cytokine profile release of vaccine-primed lymph node (VPLN) cells and tumor response. VPLN cells that had a higher IFNγ:IL-10 ratio were associated with tumor response.

in significant tumor-induced immune suppression. Because of this, we developed an animal model to examine the effect of preexistent tumor on the ability to generate tumor-primed lymph node cells for adoptive immunotherapy.

4. IMMUNE SUPPRESSION OF TUMOR-DRAINING LYMPH NODE EFFECTOR CELLS

Adoptive immunotherapy requires the in vitro generation of antitumor effector T cells from cancer patients. Tumor-associated antigen (TAA)-specific T cells are thought to be primed in the TDLNs. As described above, we use TDLNs and VPLNs as a cellular source to generate TAA-specific effector T cells. The original animal models we have established and for which we have predicated our clinical studies have utilized non-tumor-bearing mice as donors for tumor-primed lymph node cells. However, this does not mirror the clinical setting where patients bearing

tumors need to be the donors for effector cells that can be used for adoptive immunotherapy. To accurately mimic the clinical setting, we have established tumor-bearing models where in vivo tumor priming is performed to elicit pre-effector lymph node cells. In addition to the non-tumor-bearing mice, the model includes one more group of mice as donors for tumor-primed lymph node cells, who have pre-existent subcutaneous or visceral tumor when they were given tumor to prime lymph node cells.

We utilized the MCA 205 sarcoma tumor model to evaluate the effect of pre-existent tumor on the ability to elicit TDLN cells for adoptive immunotherapy. Mice were inoculated with tumor cells i.v. to establish lung metastases 3–6 days before s.c. inoculation of tumor cells in the flank. Control mice received s.c. tumor, but not i.v. tumor cells. Nine days after s.c. inoculation of tumor cells, TDLNs were harvested from the inguinal regions. The effector T cells generated from mice receiving s.c. tumor were termed TDLN. The effector T cells generated from mice receiving i.v. and s.c. tumor were termed concomitant TDLN (cTDLN). The TDLN cells were activated in anti-CD3/CD28 and expanded in IL-2. After the culture period, the antitumor reactivity of the cells was assessed in the adoptive immunotherapy of 3-day established lung metastases. As illustrated in Fig. 2, the antitumor reactivity of the cTDLN was significantly reduced on a per cell basis compared to equivalent numbers of transferred TDLN in a dose-dependent manner. The data suggest that concomitant visceral tumor induces immune suppression in the TDLNs. In a separate experiment, we found that the reduced effector function of cTDLN was dependent on timing of the lung tumor establishment. If i.v. tumor cells were injected the same day as s.c. tumor inoculation, the effector function remained, suggesting that there was no significant immune suppression induced by the lung tumors (data not shown). It indicates that the lung tumors needed to be established prior to s.c. tumor inoculation.

We also observed that the function of cTDLN is largely reduced compared to TDLN as evaluated by IFN-γ. The number of IFN-γ producing cells was significantly decreased in cTDLN compared with TDLN. This correlated with decreased amounts of IFN-γ production by cTDLN effector cells compared with TDLN cells during the activation and expansion of cells. The tumor-specific response of the activated cTDLN was significantly reduced compared with TDLN as assessed by IFN-γ production in response to irradiated tumor utilizing an ELISPOT assay. The number of tumor-induced IFN-γ spots was significantly reduced in cTDLN compared with TDLN.

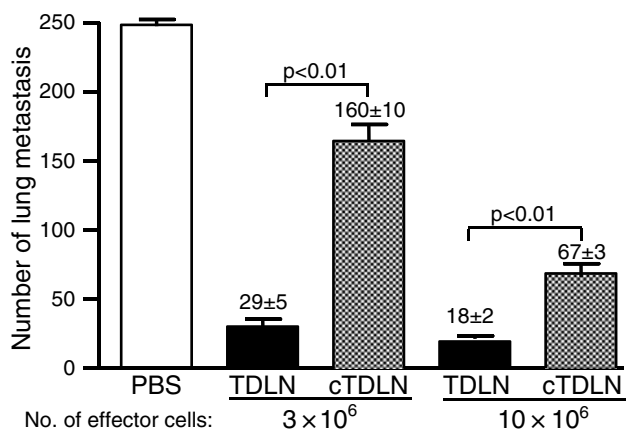

Fig. 2. cTDLN cells adoptively transferred into mice bearing lung metastases had significantly lower therapeutic efficacy than TDLN cells.

In summary, pre-established visceral or subcutaneous tumors suppress the induction of preeffector T cells in lymph nodes primed by a subsequent tumor cell inoculation in the murine model. It indicates that the anti-tumor function of effector cells generated from the cancer-bearing patient could be suppressed by the established tumors. This is very clinically relevant to the development of effective adoptive cell therapies. Blockade of suppressive mechanisms induced by established tumors can optimize the generation of antitumor reactive effector cells for immunotherapy.

5. POSSIBLE MECHANISMS OF IMMUNE SUPPRESSION

It has been shown that the tumor microenvironment is comprised of dysfunctional immune cells that have been reprogrammed by active tumor-mediated processes to defeat tumor-specific immunity in a highly effective manner. Two major mediators of poor tumor immunity are dysfunctional antigen-presenting cells (APCs) and regulatory T cells (Treg cells). Several mechanisms were described in the human cancer microenvironment that actively defeat tumor immunity *(16,17)*, including an immunopathologic role for regulatory T cells *(16)* and inhibitory B7 family members.

B7 family molecules on APCs not only provide critical positive signals that stimulate and support T cell activation, but also offer negative signals that control and suppress T cell responses *(18,19)*. Growing B7 family reaches a total of seven members including CD80, CD86, B7-H1, B7-DC, B7-H2, B7-H3, and B7-H4 *(18,19)*. B7-H1 (aka PD-L1) is a cell-surface glycoprotein that has been described to negatively regulate T cell functions by engagement with PD-1, a CD28 family member receptor. B7-H1-expressing APCs and tumor cells may mediate T cell suppression by inducing T cell apoptosis, reducing CTL cytoxicity and inhibiting DC function. Chen and colleagues reported the presence of B7-H1 protein in a wide range of human cancers *(18)*. Tumor-associated B7-H1 induces apoptosis of effector T cells and is thought to contribute to immune evasion by cancers *(20)*. DCs isolated from ovarian tumor tissues or TDLNs expressed high levels of B7-H1 *(21)*. Furthermore, blockade of B7-H1 enhanced DC-mediated T cell activation that was accompanied by downregulation of T cell production of IL-10, with a concomitant upregulation of IL-2 and IFN-γ production.

When we compare costimulatory and coinhibitory markers on DCs from TDLN and cTDLN, we observed a significant upregulation of B7-H1 with a concomitant downregulation of CD80 (data not shown) in DC from cTDLN compared to TDLN. This represents an alteration in host DC induced by the presence of established tumor. We found also that PD-1 was significantly more expressed on $CD4^+$ and $CD8^+$ cells from cTDLN compared to TDLN after activation and expansion.

We blocked the interaction between B7-H1 and PD-1 by in vitro blocking B7-H1 on cTDLN and in vivo administration of anti-B7-H1 after adoptive transfer. We observed that the treatment increased T cell IFN-γ production, enhanced the therapeutic efficacy of cTDLN. The data indicate that B7-H1/PD-1 axis plays a role in suppressing cellular responses to active immunization in the tumor-bearing host.

Treg cells are a T cell population that can functionally suppress an immune response by influencing the activity of another cell type. Different types of Treg cells are distinguished by their phenotype and function. The classic one is $CD4^+CD25^+FoxP3^+$ Treg cells. Treg cells were described as one agent of tumor-mediated antihost defense *(16,22)*. Treg cells normally mediate peripheral tolerance. However, if they are abnormally elevated in numbers or function, they have potential to perturb homeostatic immune functions or defeat a required immune response *(22)*. It was demonstrated that depletion of $CD4^+CD25^+$ T cells in a mouse model for cancer using PC61 antibody improved immune-mediated tumor rejection *(23,24)*. $CD4^+CD25^+$ T cell

depletion was also shown to boost endogenous TAA-specific immunity as well as the efficacy of active immunization *(25)*. $CD4^+CD25^+$ regulatory T cells are elevated in the peripheral blood of patients with a variety of cancers. We observed an increased percentage of $CD4^+Foxp3^+$ and $CD8^+Foxp3^+$ cells in cTDLN compared with TDLN (data not shown). After antibody activation and expansion in IL-2, the increase in $CD4^+Foxp3^+$ and $CD8^+Foxp3^+$ subpopulations persisted . TGF-β may mediate Treg cell function and/or the conversion of Treg cells from $CD4^+CD25^-$ T cells *(26–28)*. Furthermore, it is technically challenging to deplete Treg cells without hurting effector T cells in vivo. To temper Treg cell conversion and function, we treated the mice with neutralizing anti-TGF-β. Blocking TGF-β enhanced the function of T cells from both TDLN and cTDLN, as the number of IFN-γ-producing cells increased significantly. More interestingly, simultaneous blockade of TGF-β and B7-H1 synergistically promoted T cell function. More importantly, there was a synergistic increase of IFN-γ-producing cells in the presence of both antibodies that was significantly greater in cTDLN compared to TDLN cells. In adoptive transfer where exogenous blocking antibodies were also administered, the suppression of the antitumor reactivity of the cTDLN effector cells was completely abrogated when compared to TDLN effector cells.

In summary, both B7-H1/PD-1 axis and Treg cells play a role in the immune suppression we observed during in vivo priming of effector T cells in tumor-bearing mice. Blocking the B7-H1/PD-1 axis and TGF-β might provide a novel strategy to generate effector T cells in the tumor-bearing host.

6. SUMMARY

Despite the advances in the development of vaccines and immune reagents for immunotherapy, the clinical efficacy seen to date has been limited. Over the last several years, a significant body of information has evolved in defining host mechanisms that are involved with evading the immune response to TAA, thus limiting the potential therapeutic efficacy of conventional immune treatments. Future therapies will need to incorporate blockade of these immune evasion mechanisms to improve current immune therapies such as adoptive cellular therapy or vaccines.

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X The Role of Stem Cells in Cancer Metastasis

37 Cancer Stem Cells in Metastatic Melanoma

Jim B. Boonyaratanakornkit, MD, Janellen E. Smith, MD, and Ruby Ghadially, MD

CONTENTS

ABSTRACT

Melanoma remains, by far, the most deadly skin cancer and is among the most chemotherapy-resistant malignancies. While prevention and early detection improve, the incidence of melanoma continues to rise in the USA and among most fair-skinned populations worldwide. Evidence for cancer stem cells in various malignancies is rapidly increasing, and this opens a new avenue of research toward understanding the pathogenesis and effective treatment of cancer.

Key Words: cancer stem cell; melanoma; metastasis

1. CANCER STEM CELLS

Solid cancers consist of a phenotypically heterogeneous cell population with only a small proportion of cells exhibiting clonogenic capacity *(1–4)*. Two general models of how cellular heterogeneity arises in solid cancers exist. In the traditional model, all cancer cells have unlimited proliferative potential to drive tumorigenesis and metastasis, while, in the second model, only a subset of cancer cells have the potential for indefinite self-renewal and behave as tumorigenic stem cells *(5)*. The central role of a small definable subset of cancer stem cells in maintaining a neoplasm was first firmly established in hematologic malignancies through in vivo transplantation assays *(6)*. By definition, the cancer stem cell is a single cancer cell that can form tumor following transplantation (Fig. 1). The cancer stem cell does not necessarily originate from a normal stem cell but may arise from accumulated transformations in a progenitor cell *(5,7)*. Both normal stem cells and tumorigenic cells give rise to phenotypically heterogeneous progeny

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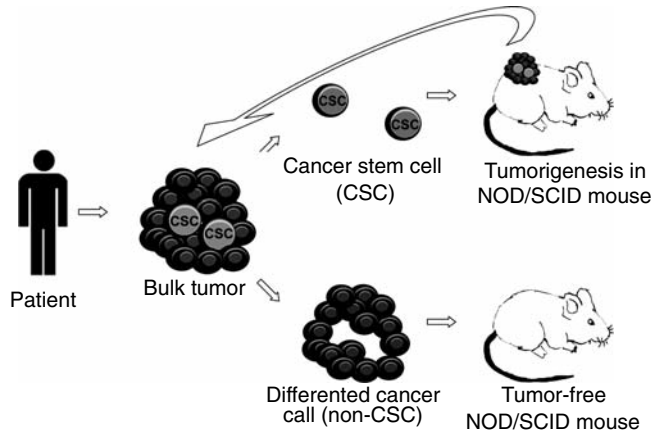

Fig. 1. The cancer stem cell is a single cell capable of forming new tumors and transferring disease upon transplantation into an immunocompromised mouse, including the nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse. Furthermore, these xenografted tumors recapitulate the phenotypic heterogeneity seen in the primary cancer. Evidence supporting the existence of a subpopulation of cancer stem cells in hematologic malignancies, brain tumors, breast cancer, and head and neck squamous cell carcinoma have been established by purifying these rare cells through flow cytometry.

with varying levels of proliferative potential and differentiation *(5,8,9)*. Based on the clonogenicity observed both in hematopoietic stem cells and leukemia cells, clonogenic leukemia cells have been described as leukemic stem cells *(5,6)*.

The application of an in vivo limiting dilution transplantation assay for acute myeloid leukemia (AML) subpopulations selected by fluorescence-activated cell sorting (FACS) provided evidence that a rare subpopulation of cancer cells could be identified that was consistently enriched for clonogenic and tumorigenic capacity, while the side population was depleted for these stem cell-like properties. Dick's group was the first to show that AML stem cells could be purified at a frequency of approximately 1 in 250,000 peripheral blood cells from patient samples by sorting for $CD34^+$ $CD38^-$ cells *(10,11)*. The $CD34^+$ $CD38^-$subpopulation was leukemia initiating, since this cellular phenotype was both necessary and sufficient for successful engraftment of human AML into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice.

Similar limiting dilution analyses on purified subpopulations have now been extended to isolated stem cells from solid tumors. A limiting dilution analysis applied to breast cancer showed that as few as 100 primary $CD44^+$ $CD24^{-/low}$ $lineage^-$ tumor cells regenerated tumors after transplantation, even with serial passaging, in NOD/SCID mice, whereas tens of thousands of cells with alternate phenotypes failed *(12)*. Recently, $CD44^+$ cancer stem cells were purified from human head and neck squamous carcinoma *(13)*. $CD44^+$, but not $CD44^-$, cancer cells, gave rise to new tumors in vivo, recreated tumor heterogeneity even after serially passaging in vivo, possessed a primitive cellular morphology, and expressed cellular markers associated with normal stem cells. Furthermore, a subpopulation of brain tumor-initiating cells were identified in human glioma in which as few as 100 $CD133^+$ cells could recapitulate tumor in NOD/SCID mice *(14)*. Beier et al. also successfully isolated a significant $CD133^+$ population from primary human glioblastomas cultured in stem cell permissive medium which displayed neurosphere-like nonadherent growth, self-renewal after serial passaging, and tumorigenesis in nude mice *(15)*. These $CD133^+$- enriched neurospheres derived from a single $CD133^+$ cell contained all three neural lineages, including astroglial, oligodendroglial, and neuronal markers. Secondary glioblastomas were devoid of $CD133^+$ expression and did not form any spheres in culture. However,

interestingly, approximately 2% of cells from adherent $CD133^-$ spheres also exhibited self-renewal after serial passaging, the potential to differentiate into all three neural lineages, and tumorigenesis in nude mice. The authors concluded that sphere-forming $CD133^+$ cells and a small subset of $CD133^-$cells represent distinct populations of cancer stem cells within the same tumor. In addition, the application of whole genome microarray analysis showed that these two cancer stem cell populations were characterized by differential gene expression profiles. The successful purification and limiting dilution analysis of $CD133^+$ tumor-initiating cells xenografted into NOD/SCID mice has also recently been reported in human colon cancer lending additional support for a model of hierarchical organization driven by cancer stem cells *(16,17)*. Taken together, these studies show that a small, predictable subset of cancer cells that is enriched for the ability to proliferate and transfer disease exists not only in hematologic malignancies but also in solid cancers.

2. MELANOMA STEM CELLS

Melanocytes are developmentally derived from the neural crest. Although melanoma is traditionally thought to arise from acquired mutations in a mature differentiated melanocyte, another model for tumorigenesis holds that melanoma originates from early undifferentiated progenitors of neural crest lineage *(18)*. Melanoma cells expressing the neuronal stem cell marker nestin were found in primary, metastatic, and experimental metastases in mice *(19)*. In addition, with respect to melanoma prognosis, the expression of melanocyte-specific markers, including Melan-A and tyrosinase, which are involved in pigment biosynthesis, is dramatically reduced or even absent in aggressive melanoma cells *(20,21)*. Reduced expression of these differentiated melanocyte markers in metastatic melanoma has been associated with a poor prognosis even after cytoreductive surgery, while expression of these genes is associated with a favorable disease outcome and longer survival *(21,22)*.

The existence of a tumorigenic subpopulation with stem cell-like properties has been discovered in metastatic melanoma by culturing nonadherent melanoma spheres in human embryonic stem cell media *(23)*. Melanoma spheroid cells exhibited self-renewal, differentiation plasticity, and the ability to drive continuous growth and tumorigenesis compared to adherent cells *(23)*. These multipotent cells persisted after serial cloning in vitro, transplantation in vivo, and could differentiate into melanocytic, adipocytic, chondrocytic, and osteocytic lineages *(23)*. Grichnik et al. sorted melanoma cell lines by Hoescht dye exclusion, a method used to purify normal stem cells based on the capacity to efflux dye, and cell size to show that small dye-low tumor cells shared many properties with normal stem cells, including a slower proliferative rate along with a greater capacity to expand in culture over time *(24)*. Moreover, Monzani et al. demonstrated, by separating $CD133^+$ from $CD133^-$ cells in human melanoma biopsies, that $CD133^+$ cells could generate melanoma tumors in NOD/SCID mice whereas $CD133^-$ cells could not *(25)*. Efforts to enrich for melanoma stem cells that are necessary and sufficient to re-create the hierarchical heterogeneity seen in melanoma and apply limiting dilution analysis after in vivo transplantation remains an active area of research.

3. CANCER STEM CELLS IN METASTASIS

The dissemination of tumor cells is a complex, multistep process usually involving a succession of specific genetic and cellular alterations that enable a cancer cell to mobilize, to survive outside of the primary tumor, and to seed and sustain growth at distant sites. The clonal selection model

of metastasis holds that acquired genetic mutations obtained late in tumorigenesis provide a selective advantage for cells that proliferate *(26)*. However, the ability to metastasize may be dependent on the cancer stem cell. Minn et al. developed a gene expression signature for breast cancer metastasis and showed retrospectively that this signature was already present in the primary tumors. Differential expression of these 54 genes was clinically predictive of metastasis in independent data sets *(27,28)*. Cells expressing the metastagenicity signature were also more tumorigenic when xenotransplanted into immunocompromised mice *(27)*. Here, a minor population of cancer cells within the heterogenous primary tumor already possessed the program to metastasize, and an overlap existed between tumorigenicity and metastagenicity.

The hierarchical model of cancer stem cells implies that long-lived tumorigenic cells mediate distant metastasis and sustain growth at distant sites, while non cancer stem cells, even if mobilized, are unable to survive, initiate, and sustain a metastatic lesion in a foreign environment. Normal stem cells and metastatic cells share similar signaling pathways to mobilize and metastasize, respectively. Both utilize matrix metalloproteinases and β-1 integrins to facilitate proliferation and migration through the extracellular matrix *(29–31)*. In addition, the chemokine SDF1 and its receptor CXCR4 play essential roles in homing and migration in both normal human stem cells and metastatic cells, particularly in breast cancer nodal metastasis and pancreatic cancer metastasis *(32–34)*.

Cancer stem cells have now been implicated not only in driving tumor initiation and growth but also in tumor metastasis, and only recently has a distinct population of cancer stem cells that can determine metastatic activity been isolated. By purifying $CD133^+$ $CXCR4^+$ tumor cells from patients with pancreatic adenocarcinoma, Hermann et al. defined a subpopulation of cancer stem cells that resided in the invasive border zone *(33)*. Depletion of this cancer stem cell pool from metastatic pancreatic cancer cell lines abolished the metastatic phenotype when orthotopically injected into athymic mice *(33)*.

Melanoma is particularly notorious for its fatal propensity to metastasize widely through lymphovascular channels to any organ *(35)*. The earliest metastases are most commonly first detected in draining sentinel lymph nodes, while lung, liver, brain, bone, and the eye are sites of hematogenous spread. Nodal status is the most important prognostic predictor of clinical outcome in melanoma *(36)*. Talmadge et al. showed that spontaneous melanoma metastases in mouse lungs were clonal in origin indicating that the metastases probably originated from different progenitor cells *(4)*. It was also shown that clones of cells derived in vitro from a parent culture of melanoma cells vary greatly in their ability to produce metastatic colonies in the lungs upon intravenous inoculation into syngeneic mice *(3)*. It has also been known for years that hematogenously derived clonal pulmonary melanoma metastases can originate from the expansion of a single tumor cell in mice *(37)*. Further, while multiple melanoma cell lines produce primary tumors in nude mice, cell lines with metastatic ability showed downregulation of HLA-DR expression and intercellular adhesion molecule 1 (ICAM-1) and marked expression of VLA-2 and epidermal growth factor receptor (EGFR) *(38)*. Repeated treatment of melanoma cells with dacarbazine can also lead to selection of more chemoresistant cell lines with enhanced tumorigenic and metastatic potential in vivo *(39)*. Taken together, these findings in melanoma are consistent with the model of tumor heterogeneity and the concept that a rare subpopulation of phenotypically distinct melanoma-initiating cells capable of mobilizing through the lymphovascular system is responsible for not only tumor maintenance but also for driving and sustaining melanoma metastases removed from the primary tumor.

4. THERAPEUTIC IMPLICATIONS OF MELANOMA STEM CELLS

Metastasis is the main cause of death for cancer patients. Metastatic melanoma is, in general, incurable with a median survival of 7.5 months *(35)*. The identification of a subpopulation of melanoma stem cells would have significant implications for revolutionizing current therapeutic modalities *(5)*. As stem cells rarely divide, express high levels of drug transporters to export chemotherapeutic agents, and activate DNA repair pathways, many chemotherapies and radiotherapies are insufficient in clearing the very cell that drives tumorigenesis and metastasis *(40–42)*. Currently available drugs and surgery are designed to shrink the bulk tumor but often leave the cancer stem cell untouched *(5)*. Instead, immunotherapy specifically against the cancer stem cell, inhibiting self-renewal pathways like hedgehog and Wnt/β-catenin signaling, inducing differentiation, blocking drug transporters, or blocking key chemokine receptors instrumental in metastasis would offer new avenues for effective melanoma therapy (Fig. 2). Functional genomics with whole genome expression profiling of purified cancer stem cells would provide insight into the biology that differentiates the cancer stem cell from the bulk tumor and into specific therapeutic targets. Targeted killing of the melanoma stem cell, particularly the metastatic stem cell, would provide more durable responses and even cures for patients with metastatic disease.

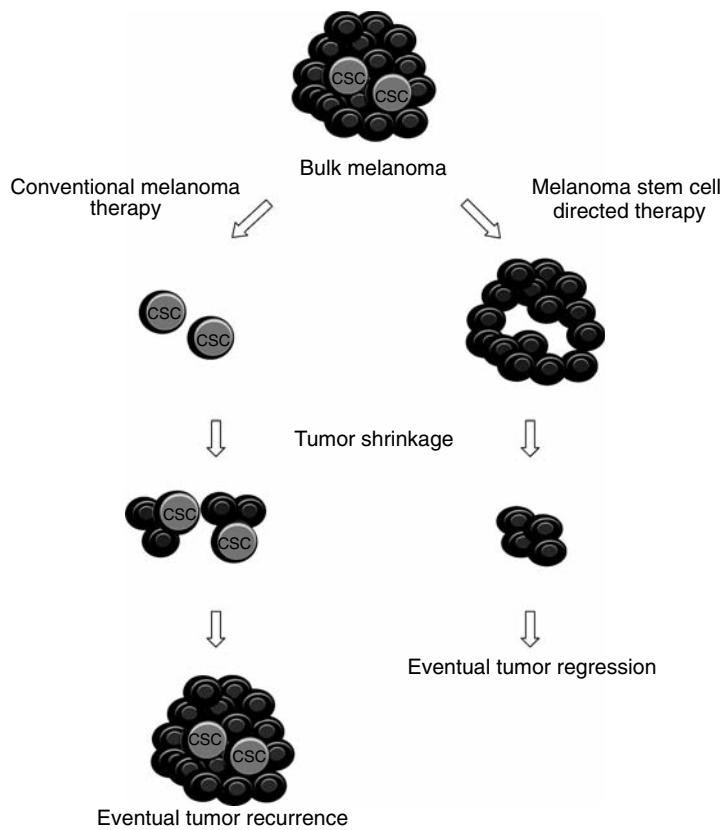

Fig. 2. Conventional therapies against cancer rely on shrinking the bulk tumor and often leave the chemoresistant and radioresistant cancer stem cell untouched. This strategy often allows tumor recurrence. In contrast, a cancer stem cell-targeted therapy would specifically kill the cell responsible for driving tumorigenesis and metastagenesis and lead to long-lasting tumor regression.

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38 Implications of Cancer Stem Cells for Tumor Metastasis

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Contents

ABSTRACT

Metastasis is the dissemination of cancer cells from primary tumors to distant sites. This phenomenon accounts for greater than 90% of deaths associated with cancer *(1)*. Dissemination of cancer cells is a complex process involving a sequence of diverse functional steps, such as detachment from the primary tumor tissue, migration and homing to a different anatomical site, and survival and growth into a metastatic cancerous lesion. Traditional observations suggest that this phenomenon is mediated by late-stage genetic alterations which program cancer cells for active mobilization and distant-site engraftment.

With the discovery that solid tumors may be driven in their growth by subsets of cancer cells selectively endowed with tumor-initiating capacity—also termed *cancer stem cells* (CSCs)—tumor progression has come under new investigation. These cells exhibit properties reminiscent of normal tissue stem cells: they are a phenotypically unique subset of cells capable of recapitulating the morphologic and phenotypic diversity of the original tumor when serially transplanted in immunodeficient mice. Given their preferential capacity to self-renew and form new tumor lesions, these cells have been postulated to play a key role in metastatic cancer. Moreover, gene expression studies have recently demonstrated a high correlation between CSC gene expression profiles, the risk of metastasis, and patient survival.

In this chapter, we aim to discuss the implications of the CSC theory in metastatic cancer. We will begin by introducing CSCs, drawing functional parallels between them and normal stem cells. We will then discuss their biology and highlight their clinical relevance. Further study of

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CSCs is important as they may hold potential for specific therapeutics aimed at reducing or eliminating the tumor load by eradicating the source of the tumor, the cells that initiate and sustain cancerous growth.

Key Words: cancer stem cells; patient prognosis; metastasis-free survival; microarray gene expression profiling; immunohistochemistry

1. STEM CELLS: GENERAL PRINCIPLES

Stem cells are defined as cells with two inherent functional characteristics: (1) the ability to differentiate into specialized progenies of functionally committed daughter cells (differentiation) and (2) the ability to reproduce themselves, in order to maintain constant their populations over time (self-renewal). Stem cell biology has been defined and characterized most strongly in the hematopoietic system. In recent years, however, the discovery of stem cells has expanded beyond the hematopoietic system to include isolation and characterization of breast, prostate, heart, lung, and brain stem cells to name a few *(2–7)*. Isolation of these cells allows, for the first time, their detailed functional study and their manipulation for potential therapeutic use in regenerative medicine.

Isolation of normal stem cells has been primarily achieved exploiting their unique profile of surface marker expression. Isolation techniques are usually based on immunoselection procedures: stem cells are labeled with monoclonal antibodies directed against appropriate surface markers and conjugated to either fluorescent compounds or magnetic beads; once tagged by the antibodies, stem cells can be purified by flow cytometry (i.e., fluorescence-activated cell sorting or FACS) or by magnetic separation.

Among the hallmarks of stem cells is their ability to undergo different types of cell division, including both symmetric and asymmetric divisions. In an asymmetric division, a stem cell divides to produce a new stem cell and a differentiated progenitor. In symmetric divisions, a stem cell divides to produce two functionally identical cells, either both stem cells or both differentiated progenitors. Stem cells therefore walk a fine line between self-renewal and differentiation, a key and delicate balance under the control of both intrinsic cellular traits (either genetic or epigenetic) and external signals from the surrounding environment *(8–10)*.

Due to their self-renewal capacity, stem cells are usually characterized by an extraordinary expansion and proliferation potential. A classical example of this property is provided by stem cells of the hematopoietic system, where one single hematopoietic stem cell (HSC) is capable of repopulating the blood system of a lethally irradiated mouse and sustain the production of all blood lineages for the lifetime of the animal *(11)*. Despite their huge expansion potential, however, stem cells are usually rare populations, typically representing a very low percentage of the total number of cells found in a specific tissue (e.g., it is calculated that HSCs represent as little as 0.01% of the total cells found in the bone marrow) *(11,12)*.

2. CANCER STEM CELLS: DEFINITION AND IDENTIFICATION

The notion that cancer can be interpreted as a stem cell disease was first introduced in the case of human myeloid leukemias. A first line of evidence was provided by Philip Fialkow and colleagues, who showed that several neoplastic disorders of the blood system, including both chronic and acute myelogenous leukemias, where characterized by the expansion of monoclonal cell populations which contained different cellular lineages *(13,14)*. These observations were followed by a seminal set of studies by the group led by John Dick, which showed that, in several

types of human acute myeloid leukemia, the capacity to engraft in immunodeficient mice is restricted to a minority subset of cells that share a surface marker profile similar to that of HSCs ($CD34^+CD38^-$) and have thus been termed leukemic stem cells *(15,16)*. Recently, the concept that tumor growth could be sustained by a subset of cancer cells with unique functional properties was extended first to breast cancer *(17)* and subsequently to various other types of human solid tumors *(18–27)*. Serial transplantation assays have demonstrated the ability of these cells to self-renew and give rise to phenotypically heterogeneous tumor tissues, containing diverse phenotypical and morphological subsets of cancer cells. These cells have thus been termed *cancer stem cells* (CSCs) for functional similarity to normal tissue stem cells *(28)*.

As in the case of normal stem cells, CSCs are characterized by a specific surface marker expression profile and can be isolated using FACS or other immunoselection procedures, such as magnetic beads. In most cases, CSCs make up a small percentage of the total cancer cells within a specific tumor mass. When purified and xenografted in immunodeficient mice (e.g., NOD/SCID), human CSCs are selectively endowed with the capacity to form new tumors, and are able to fully recapitulate the original cancer they were derived from, both histologically and in terms of the phenotypic repertoire of cancer cell populations that are contained within the tumor tissues. Because of this functional resemblance to stem cells, it has been suggested that CSCs might share with normal stem cells biological similarities, especially with regard to biochemical pathways controlling self-renewal.

The surface marker phenotype exploited to purify CSCs differs from tumor type to tumor type, although some markers can be successfully exploited across different forms of human cancer. The first class of solid tumor CSCs was identified in breast cancer, where it was shown that the tumorigenic population of breast-CSCs (Br-CSCs) is characterized by a $CD44^+CD24^{-/low}$ surface marker profile *(17)*. Interestingly, differential expression of CD44 can be exploited for the isolation of CSCs in several human epithelial tumors, as in the case of prostate ($CD44^+$) *(18,23)*, head and neck ($CD44^+$) *(24)*, and pancreatic cancer ($CD44^+CD24^+EpCAM^+$) *(21)*. Recently, a similar approach has been successfully applied also to the study of colorectal cancer, where CSCs can be purified using CD44 in combination with other two surface markers: EpCAM and CD166 ($CD44^+/CD166^+/EpCAM^{high}$) *(19)*. Another marker that is frequently utilized to isolate CSCs from different tumor types is CD133, also known as Prominin-1. CD133 is expressed on normal hematopoietic *(29)*, neural *(7)*, and prostate epithelial stem cells *(4)* and can be exploited to isolate CSCs from several brain, colon, and pancreatic cancers *(22,25,30)*.

Some authors have raised caution in the interpretation of studies that investigate the existence of CSCs based on transplantation studies, suggesting that species-specific barriers (mouse vs. human) may exist with regard to engraftment and tumor-initiating capacity of different cancer cell subpopulations *(31)*. It is argued that, in xenotransplantation experiments, different subpopulations of human cancer cells might differ in their tumorigenic capacity not because of intrinsic differences in their differentiation state and self-renewal capacity, but because of differences in their capacity to survive in the recipient mouse environment *(31)*. To address this concern, we sought to validate the CSC model in a syngeneic mouse cancer system, where no species-specific barriers to transplantation would exist *(32)*. Using a transgenic mouse cancer model (i.e., mouse mammary tumors developed in MMTV-*Wnt-1* transgenic mice), our laboratory recently demonstrated the existence of a mouse Br-CSC population in spontaneous de novo mammary tumors. In this mouse breast cancer model, the tumorigenic capacity was restricted to a subpopulation of cells characterized by the $Thy1^+CD24^+$ surface marker phenotype *(32)*. In this same study, a comparison of the gene expression profiles of $Thy1^+CD24^+$ Br-CSCs with their nontumorigenic counterparts indicated that Br-CSCs tended to overexpress genes that are characteristic of basal cells of the mammary epithelium, such as basal cell keratins (i.e., Krt5, Krt14, Krt17), or that are involved in stem cell function (i.e., Notch-4, Bcl6b). On the other hand,

nontumorigenic cells tended to overexpress genes implicated in luminal epithelial cell function, such as luminal cell keratins (i.e. Krt18, Krt19), whey acidic protein (Wap), lactotransferrin (Ltf), claudin 1 (Cldn1), and the luminal epithelial marker Elf5. Interestingly, the expression pattern of these genes mirrored their expression in normal mammary stem cells and their progenitors *(6)*. These observations suggest that differences in the tumorigenic capacity of CSCs as opposed to their nontumorigenic counterparts are probably best explained by differences in the inherent differentiation status of the different cancer cell subsets.

3. CANCER STEM CELLS: ROLE IN TUMOR METASTASIS

Because of their ability to self-renew, and thus to sustain long-term tumor growth, it has been suggested that CSCs might be implicated in generating and maintaining tumors at distant sites *(28)*. An indirect support to this hypothesis has recently come from a series of investigations based on the transcriptional profiling of CSCs which have correlated CSC gene expression patterns with the risk of distant-site metastasis and overall patient survival *(33)*. Based on a comparison of the gene expression profile of human Br-CSCs ($CD44^{+}CD24^{-/low}$) with that of whole normal mammary breast epithelium, a pool of 186 genes was identified to vary in expression levels between these two populations. The 186 genes and their expression levels were used to generate an "invasiveness gene signature" (IGS) and were evaluated for association with both overall and metastasis-free survival in patients with breast cancer. A positive correlation with the IGS was associated with poor prognosis while a negative correlation was associated with good prognosis. The 10-year overall survival rate for the group with a negative correlation to the IGS was placed at 98%, while the rate for the group with a positive correlation was placed at 62% (Fig. 1 and Color Plate 27, Panel A). Most interestingly, the differences in overall survival were tightly correlated to differences in metastasis-free survival: the 10-year metastasis-free survival rate for the group with a negative correlation to the IGS was placed at 82%, while the rate for the group with a positive correlation was placed at 54% (Fig. 1, Panel B). The association of the IGS with patient outcome was independent of other standard clinical and pathological criteria, such as primary tumor size or the concurrent presence of lymph node metastases, and remained statistically significant in multivariate analysis regardless of other classical prognostic factors such as age, tumor differentiation, and estrogen receptor status.

Interestingly, the prognostic role of the IGS was not only independent of other standard prognostic factors, but also of other, previously described gene expression signatures endowed with prognostic capacity in breast cancer, such as the wound response (WR) signature, a 512-gene signature obtained from the transcriptional profiling of serum-stimulated fibroblasts *(34)*. When tested in multivariate analysis, the IGS and the WR signature performed independently, with a hazard ratio for the 5-year risk of metastasis of 1.3 ($p = 0.001$) and 1.2 ($p = 0.003$), respectively. When used in combination, the IGS and WR signature performed in a synergistic way: at 10-year follow-up the risk of metastasis for patients whose tumors scored as negative for both signatures, positive for only one signature or positive for both signatures was 20, 31, and 53%, respectively (Fig. 2 and Color Plate 28). These observations suggest that the association of the IGS with the risk of metastasis is representative of a novel and unique biological phenomenon, independent of those portrayed by other signatures previously described in the scientific literature *(35)*. Even more striking, the prognostic power of the IGS was not restricted to breast cancer patients, but extended to different tumor types such as lung cancer, medulloblastoma, and prostate cancer *(33)*. Among patients affected by these forms of cancer, tumors whose transcriptional profile more closely correlated with the IGS were associated with overall or relapse-free survival rates of less than 50%. Taken together, these observations

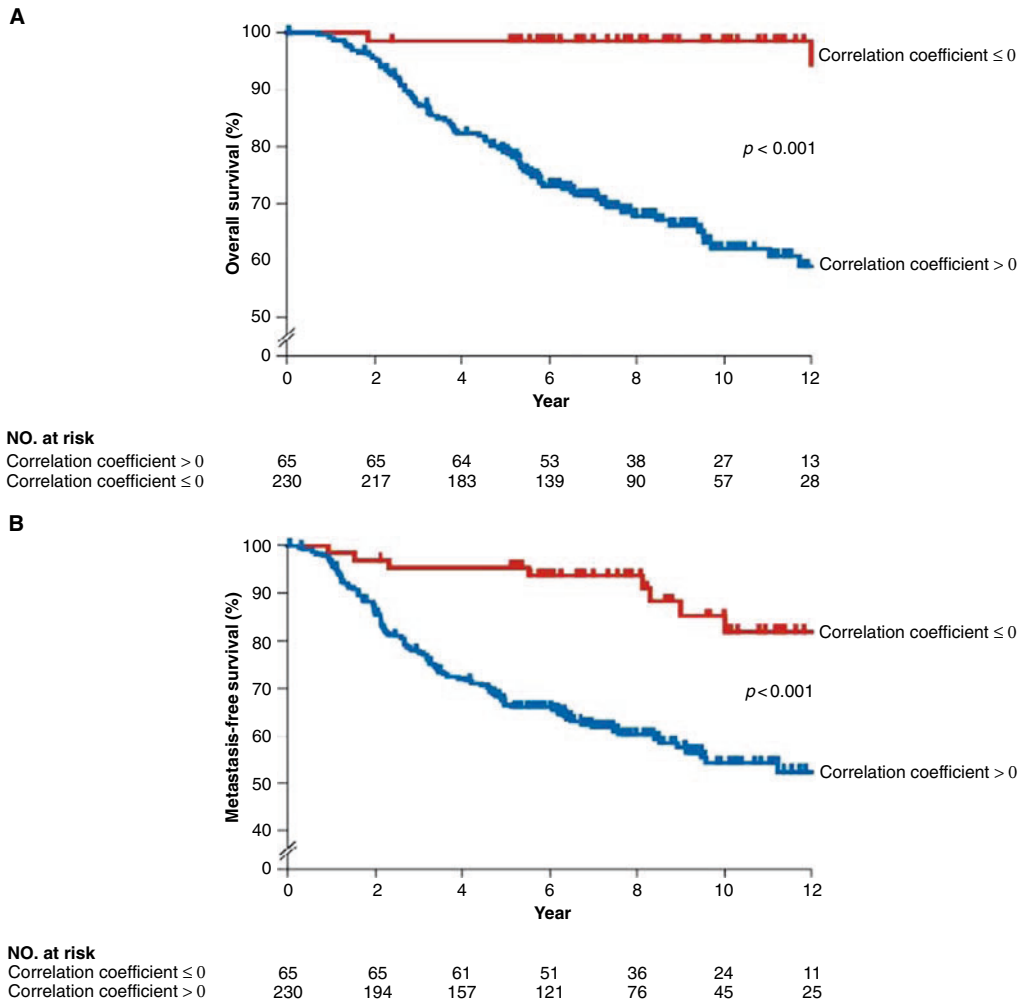
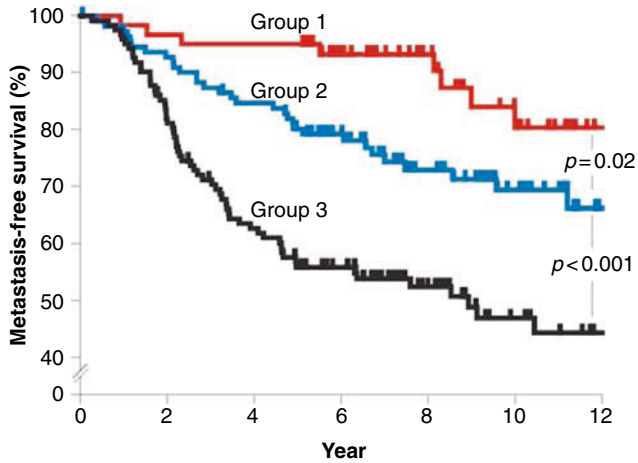


Fig. 1. Association between Br-CSC gene expression profiles and prognosis of breast cancer patients. A Pearson correlation coefficient was calculated for the correlation between a gene signature obtained from Br-CSCs (the "invasiveness gene signature" or IGS) and each of the 295 tumors included in a publicly available breast cancer patient database (the Netherlands Cancer Institute database) on the basis of the expression values of the 186 genes that are included in the gene signature. Patients were separated into two groups according to the correlation values, with 0 used as the threshold. Kaplan–Meier survival curves for the two groups were compared, with overall survival (Panel **A**) and metastasis-free survival (Panel **B**) as the clinical end points. Patients with tumors with a gene expression pattern that was similar to the IGS (correlation coefficient, >0) had worse outcomes than those with tumors with a gene expression pattern that was not similar to the IGS (correlation coefficient,≤0). Reproduced with permission from Liu et al., *N Engl J Med*, 2007; 356:217–226. (*see* Color Plate 27)

suggest that analysis of CSCs could provide important insights in the processes of tumor relapse and metastasis and that these processes could be associated to a core set of CSC functional properties that are shared across different tumor types.

Evidence for shared molecular CSC features across different tumor types, and even animal species, is further provided by a study recently performed in our laboratory on MMTV-*Wnt-1* mouse breast cancers *(32)*. In this study, the gene expression profile of MMTV-*Wnt-1* Br-CSCs was compared to that of their nontumorigenic counterparts and, again, the differentially



No. at risk							
Group 1	61	60	59	48	33	22	10
Group 2	112	104	94	72	49	35	17
Group 3	112	99	74	59	36	21	13

Fig. 2. Metastasis-free survival of breast cancer patients after stratification based on the combined use of the IGS and the WR signature. A Pearson correlation coefficient was calculated for the correlation between each of the two signatures (IGS and WR) and each of the 295 tumors included in the Netherlands Cancer Institute database. Group 1 included patients with a negative correlation to both the IGS and the WR signature. Group 2 included patients with a positive correlation to either the IGS or the WR signature. Group 3 included patients with a positive correlation to both the IGS and the WR signature. The 10-year metastasis-free survival of the three groups was 80, 69, and 47%, respectively. Reproduced with permission from Liu et al., *N Engl J Med*, 2007; 356:217–226. (*see* Color Plate 28)

expressed genes used to generate a gene expression signature. Based on the human counterparts of the differentially expressed mouse genes, a second 168-gene signature was created and utilized to stratify breast cancer patients from two different databases, one from the Netherlands Cancer Institute (295 patients) and one from the Karolinska Institute/Hospital (395 patients). In the first case, the 12-year overall survival rate for patients with a negative correlation to the 168-gene signature was 75%, as opposed to 49% for those with a positive correlation ($p < 0.0003$). In the second case, the 10-year overall survival rate for patients with a negative correlation was 85%, as opposed to 66% for those with a positive correlation ($p < 0.0001$).

Similar results are provided by yet another set of studies correlating the expression of genes involved in stem cell biology with both risk of metastasis and overall survival of cancer patients, such as in the case of Bmi1. Bmi1 is a member of the *Polycomb* group of transcriptional repressors and is very well known to play an important role in both stem cell and cancer biology. Bmi1 is necessary for the maintenance of self-renewal in hematopoietic and neural stem cells *(36,37)* and for the long-term maintenance of tumor growth in mouse models of acute myeloid leukemias *(38,39)*. It is presumed that Bmi1 exerts this role through the transcriptional silencing of the *Ink4a*/*Arf*tumor-suppressor locus *(37,40)*. Recent evidence from a study on head and neck cancer shows that Bmi1 is preferentially expressed in the $CD44^+$ cell subset, which is known to contain the CSCs *(24)*.

Investigations on the clinical significance of Bmi1 expression in primary tumor tissues suggest that high levels of Bmi1 gene or protein expression are frequently associated with a higher risk of metastasis and with shortened relapse-free survival in different types of human cancer, including breast cancer, prostate cancer, and melanoma *(41–43)*. A similar conclusion

is also supported by a gene expression profiling study by Glinsky et al., where the comparison of the transcriptional profiles of neural stem cells from $Bmi1^{+/+}$ versus $Bmi1^{-/-}$ mice was exploited to generate an 11-gene transcriptional signature endowed with prognostic capacity in many forms of human cancer *(44)*.

An additional line of evidence in support of the role of CSCs in tumor metastasis is provided by studies that investigate the expression and distribution in primary tumor tissues of surface markers used for CSC isolation, such as CD166/ALCAM. Our laboratory has recently shown that CD166 is a marker of human colorectal CSCs *(19)*. Interestingly, Weichert et al. have shown that CD166 overexpression in primary colorectal tumors is associated with shortened patient survival *(45)*. An association between CD166 expression and tumor invasiveness has also been very well documented in malignant melanoma, where CD166 expression is restricted to the vertical growth phase of primary lesions and is directly correlated to tumor thickness *(46,47)*. Moreover, recent data indicate that CD166 expression in malignant melanoma is tightly correlated to the expression of ABCB5, a newly described melanoma CSC marker *(26,48)*.

The main conceptual implication of all these studies, which provide evidence that CSC content correlates with the risk of metastasis, is that CSCs might be the cells directly responsible for initiating the process of metastasis. Traditionally, metastasis is modeled as the consequence of late genetic changes that produce cancer cell clones with selective survival advantage and flexible tissue tropism, yielding the potential for growth in distant sites. The CSC model suggests that CSCs may be the cells responsible for metastasis due to their capacity for self-renewal and long-term proliferation. The two models are not necessarily mutually exclusive; however, they make slightly different predictions. The classic model envisions metastases as expansions of individual, very specific cancer cell clones, endowed with special genetic and functional properties. Thus, in classical models, tumor metastases are envisioned as substantially different from their parent primary tumors (Fig. 3, Panel A). On the contrary, the CSC model envisions metastases as "carbon copies" of the primary tumors, initiated and sustained in their growth by CSCs and thus reproducing in a distant site the morphogenetic and differentiation programs observed in the parent tumor tissue (Fig. 3, Panel B). Evidence in support of this second scenario comes from studies that systematically compared primary tumors with paired samples of autologous metastases: these investigations revealed that metastases tend to maintain a very high degree of similarity with their corresponding primary tumors, both in terms of histopathological features *(49,50)* and overall gene expression profiles *(1,51,52)*.

A series of important insights on the possible role of CSCs in the formation of metastases comes also from a set of very accurate and comprehensive histopathological investigations by Brabletz and coworkers *(50)*. These authors have analyzed the structural organization of colorectal tumor tissues with special attention to the pattern of expression of β-catenin, a molecule involved in the signal transduction of the *Wnt*biochemical pathway and in the control of self-renewal in multiple types of stem cells. These studies have shown that expression of β-catenin follows a recurrent pattern, both in primary tumors and distant-site metastases: while cancer cells that make up the central portion of the tumor express β-catenin predominantly on the membrane and in the cytoplasm, cells on the periphery, especially on the invasive front of the tumor, express β-catenin predominantly in the nucleus *(49)*. Remarkably, acquisition of nuclear β-catenin expression correlates with the acquisition of a set of features that are traditionally associated with the acquisition of metastatic capacity, such as loss of E-cadherin and acquisition of an undifferentiated, invasive morphology, a phenomenon frequently referred to as epithelial to mesenchymal transition (EMT). These observations suggest that CSCs might correspond to the cells at the invasive front of cancer tissues undergoing EMT, and thus to the cells that can acquire movement capacity and cause metastasis.

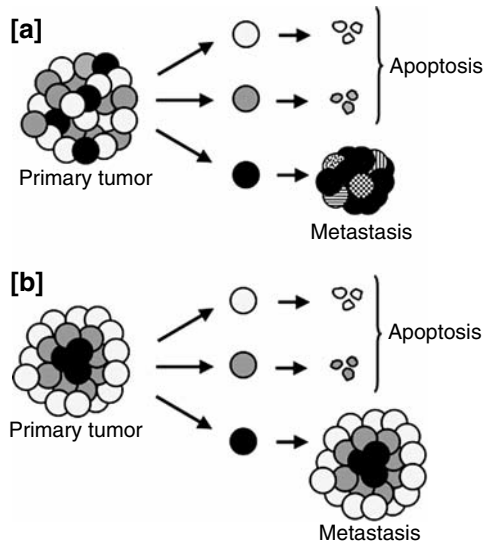


Fig. 3. Implications of the CSC model on the origin and biology of metastases. (**a**) According to classical models of cancer, tumors are composed of polyclonal populations of cells originating from different genetic mutations. Different cellular clones are endowed with different functional properties. In this scenario, metastatic dissemination is a property of a subpopulation of selected clones (*dark cells*), which have acquired the capacity to migrate and establish in distant tissues. Metastases are thus predicted to result from the expansion of a specific subclone of cells, which is characterized by a distinctive set of genetic aberrations (*dark cells*) and which can accumulate additional divergent mutations (*striped and variously patterned cells*). (**b**) The CSC model assumes that only the CSC subpopulation (*dark cells*) is capable of long-term self-renewal, and thus is the only cellular subset that is capable to sustain the growth of a metastasis. In the CSC model, metastatic cancer tissues undergo differentiation programs that closely resemble those observed in the corresponding primary tissues. Reprinted with permission from the *Annual Review of Medicine,*Volume 58 © 2007 by Annual Reviews, www.annualreviews.org.

This concept has recently been reinforced by a study on pancreatic cancer, which suggests that, within the same tumor tissue, pancreatic CSCs might exist in two different phenotype subsets, $CD133^+CXCR4^{neg}$ and $CD133^+CXCR4^+$, and that only one of the two, the $CD133^+CXCR4^+$, is actually capable of metastasis *(30)*. This observation introduces a novel concept, i.e., that metastasis might be driven by a unique subset of CSCs a subpopulation among a subpopulation of the cancer, which acquires migratory capacity through the expression of a chemokine receptor (CXCR4). However, it is still uncertain whether the $CXCR4^+$ population represents a clonal subset of CSCs or whether CXCR4 expression is induced by microenvironmental stimuli *(53)*.

Interestingly, expression of CXCR4 can be induced by hypoxia through the induction of the hypoxia inducible factor (HIF) *(54,55)*. In addition to inducing CXCR4, HIF regulates several pathways involved in the biology of both stem cells and metastasis: (1) it upregulates the expression of lysyl oxidase (LOX), thus increasing the ability of tumor cells to attach to foreign sites and establish new lesions *(56)*; (2) it decreases levels of E-cadherin, thus facilitating the acquisition of an EMT phenotype by cancer cells *(57,58)*; and (3) finally, it upregulates the expression of the MET proto-oncogene which is known to be one of the key regulators of cancer cell invasiveness and metastasis *(59)* and is increasingly regarded as a key player in the control of several stem cell functions, including self-renewal *(60)*.

4. CONCLUSIONS

Increasing experimental evidence supports the concept that tumor origin, growth, and progression can be linked to a unique subset of cancer cells with striking similarities to normal tissue adult stem cells. These cells, also known as *cancer stem cells* (CSCs) are the only cells capable of sustaining the growth of a secondary tumor lesion when transplanted in immunodeficient animals.

Although the connection between CSCs and the process of metastasis remains to be fully elucidated, several indirect lines of evidence indicate that CSC content within a tumor tissue is correlated to increased risk of metastasis and reduced overall survival of cancer patients.

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39 Prostate Cancer Stem Cells and Their Involvement in Metastasis

Hangwen Li, BS and Dean G. Tang, PhD

Contents

ABSTRACT

Several solid tumors have now been shown to contain stem cell-like cells called cancer stem cells (CSCs). These cells, although generally rare, appear to be highly tumorigenic and may be the cells that drive tumor formation, maintain tumor homeostasis, and mediate tumor metastasis. In this chapter, we first summarize our current knowledge of stem/progenitor cells in the normal human prostate, an organ highly susceptible to hyperproliferative diseases such as benign prostate hyperplasia and prostate cancer (PCa). Then we provide our insight on how a CSC should be defined. We further review the evidence of CSCs in PCa. Along with our discussion, we present several methodologies that can be potentially used to identify putative tumor-initiating CSCs. Finally, we discuss the involvement of CSCs in metastasis and the potential implications of the CSC model in helping us to understand the progression of prostate carcinogenesis and metastasis and to design novel diagnostic, prognostic, and therapeutic approaches.

Key Words: prostate cancer; cancer stem cells; tumor progenitor cells; metastasis; metastatic cancer stem cells

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1. INTRODUCTION

Prostate cancer (PCa) is the most common cancer in men in North America *(1)*. Almost 30,000 PCa-related death was reported alone in the USA in 2006 *(2)*. Despite recent advances in diagnosis and therapeutic techniques of surgery and radiation that are becoming increasingly effective in controlling the primary tumor, the survival rate of PCa patients has not significantly improved due to the posttreatment recurrence of metastatic diseases, which account for most of cancer death *(3)*. This stagnancy of effective therapy for PCa metastasis to a large extent is due to poor understanding of the molecular mechanisms for the complex multistage metastatic process including invasion, survival, arrest in the bloodstream and metastatic colonization *(3)*. The recently revived cancer stem cell (CSC) theory seems to shed light on better understanding of tumor progression. In fact, stem-like cells or CSCs have been identified in several solid tumors including those in the breast, brain, colon, and pancreas *(4–7)*. However, the question of whether CSC theory fits to solve metastasis puzzles, especially the origin of metastasis remains to be unanswered.

2. NORMAL PROSTATE STEM/PROGENITOR CELLS

Stem cells (SCs) are characterized with two fundamental biological traits, self-renewal and multipotency, as have been established from studies of hematopoiesis *(8,9)*. The adult SCs, or tissue SCs, are undifferentiated cells with the self-renewal ability and the capacity to differentiate into different lineages of progeny and even to reconstitute an organ or regenerate the damage tissues, such as a single hematopoietic SC (HSC) being able to reconstitute the whole blood and rescue a lethally irradiated mouse. Based on their self-renewal abilities, HSCs can be defined as long-term (LT) HSCs, short-term (ST) HSCs, and multipotent progenitor (MPP) cells *(8)*. In most other organs (including prostate) the lineage relationship of SC development has not been clearly defined; therefore, the term 'stem/progenitor cells' is often used.

The prostate is a male secondary sex organ whose primary function is to produce seminal fluid for reproduction. The prostate is an epithelial glandular organ containing three types of cells: basal and luminal epithelial cells and neuroendocrine cells *(10–14)*. Basal cells form the basal layer along with the basement membrane; luminal cells are above basal cells and secret prostatic proteins into the luminal space; and neuroendocrine cells generally transverse the basal and luminal layers and secret neuroendocrine peptides that support epithelial growth. The prostate is an androgen-regulated organ and classic androgen-cycling experiments in rodent prostate demonstrate that the organ can undergo multiple rounds of castration-induced regression and testosterone-induced regeneration, suggesting the presence of regenerative SCs *(12,13)*. The observations that most of the survived cells upon castration were basal cells led to the traditional hypothesis that the basal layer contains prostate stem/progenitor cells *(12)*. Several other pieces of evidence provide support for this hypothesis. *First*, some important molecules in regulating SC self-renewal and survival such as p63, hTERT, and Bcl-*2* are localized in the basal layer *(10,15)*. *Second*, there has been some evidence that basal cells can differentiate to luminal cells (reviewed in *[13]*). *Third*, if the basal layer marker p63 is knocked out, male mice were born without the prostate *(16)*. It seems that cells in the basal layer can produce transit-amplifying cells, which then give rise to terminally differentiated luminal cells. On the other hand, long-term BrdU labeling (i.e., label-retaining cells or LRC) experiments indicate that the LRCs are localized in both basal and luminal cells in the proximal region that is thought to be the prostate SC niche *(17)*, suggesting that prostate SCs (especially in the rodents) may not be restricted to the basal layer.

In human, several candidate populations of prostate stem/progenitor cells have been identified using cell surface markers including CD44, $\alpha 2\beta 1$, or CD133 (reviewed in *[10]*; for mouse prostate stem/progenitor cells, see *[13]* for review). CD44 is expressed on most basal cells and is related to SC self-renewal and tumor metastasis *(18,19)*. Recently, the $CD133^+$ cells, enriched in the $CD44^+\alpha 2\beta 1^{hi}$ basal cell population, have been shown to possess the high proliferative potential in vitro and ability to produce prostate glandular structure when transplanted in mice *(20)*. Interestingly, all primary normal human prostate (NHP) cells express basal cell markers such as CD44, $\alpha 2\beta 1$,CK5, hTERT, and p63 but not luminal markers, suggesting that these primary cells may still contain some prostate stem/progenitor cells *(21)*.

3. CSCs: RESEMBLANCE TO NORMAL SCs AND SEEDS OF TUMOR DEVELOPMENT

It was reported 50 years ago that a minor subset (0.01–1%) of cancer cells acutely isolated from tumors had the ability to regenerate a clonal growth or a tumor, suggesting that these rare cells may represent tumor SCs (reviewed in *[22]*). The pioneering work by John Dick and his group on acute myelogenous leukemia (AML) revived studies on CSCs *(23)* and putative CSCs have so far been reported in multiple solid tumors *(4–7)*. Two basic principles are shared by these studies: (1) all these presumptive CSCs are identified using normal stem/progenitor cell markers; and (2) initiation of serially transplantable tumors in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice has been used as the gold standard to functionally define the reported CSCs (reviewed in *[10]*). Since most of the reported CSCs have not been directly shown to possess multipotent differentiation ability, 'tumor-initiating cells' may be more appropriate (than CSCs) in defining these stem-like cells.

It has long been postulated that CSCs might have derived from the transformed normal SC counterparts, for several reasons. *First*, CSCs seem to generally share surface antigenic markers with the normal SCs, implying that they come from the same lineage. *Second*, the unique self-renewal capacity of SCs would allow them to accumulate multiple mutations required for malignant transformation. Presumably, it will be more difficult (or would take more mutations) for a terminally differentiated cell to attain the self-renewal ability. *Third*, some human malignancies such as chronic myelogenous leukemia (CML) seem to have a SC origin; for example, the CML signature mutation Philadelphia chromosome is present in HSCs (reviewed in *[13]*). On the other hand, progenitor cells, which are more abundant than the SCs, could conceptually acquire the self-renewal properties and become CSCs. Indeed, in CML blast crisis, GM progenitor cells activate the β-catenin signaling pathway and function as leukemic SCs *(24)* and overexpression MLL-AF9 successfully induces leukemia from committed myeloid progenitors *(25)*. Yet another potential source of CSCs could be bone marrow-derived cells (BMDCs), which may directly undergo transformation *(26)*, or first undergo chronic inflammation-induced 'transdifferentiation' into epithelial cells followed by transformation *(27)*, or first fused with neoplastic epithelium *(28)*. How much BMDCs contribute to the development of most human tumors remains to be determined and it is quite possible that each type of tumors or even each individual patient tumor might have a unique CSC population.

4. HUMAN PROSTATE CSCs: PCa-INITIATING CELLS

Potential PCa-initiating cells can be identified by several different methods *(10)*.

4.1. Surface Markers

CD44, a cell surface adhesion molecule, has been utilized as the positive surface marker to identify tumor-initiating cells in breast *(4)* and pancreatic *(7)* cancers. In prostate, some CD44-expressing prostate epithelial cells can differentiate into prostate-specific antigen (PSA)-producing cells *(16)*. Our comprehensive studies using several xenograft tumor models revealed that highly purified $CD44^+$ PCa cells are more proliferative, clonogenic, tumorigenic, and metastatic than the corresponding $CD44^-$PCa cells *(18)*. Interestingly, when the other two normal SC markers, ABCG2 and $\alpha 2\beta 1$, are used in PCa studies, we have found that both $ABCG2^+$ and $\alpha 2\beta 1^+$ subsets do not demonstrate more tumorigenic potential than the corresponding $ABCG2^-$ and $\alpha 2\beta 1^-$ subpopulations *(19)*. However, the $CD44^+\alpha 2\beta 1^+$ double-positive cells are highly tumorigenic and even exhibit slightly higher tumorigenic potential than the $CD44^+$ single-positive cell population. These observations suggest that the $CD44^+$ population may contain both CSCs and tumor progenitor cells, both of which can initiate tumor development and that the true prostate CSCs, localized in the $CD44^+$ cell population, remains yet to be identified. In support of this latter possibility, the $CD33^+\alpha 2\ \beta 1^{hi}CD44^+$ PCa cells from primary patient tumors have been reported to possess CSC properties in vitro *(29)*.

4.2. Side Population Technique

Side population (SP) is a flow cytometry-based technique taking advantage of preferential expression of the ABC family transporters in SCs that mediate the exclusion of nuclear dye Hoechst 33342 and this approach has been widely used to enrich for normal and cancerous SCs (reviewed in *[10,30,31]*). In PCa, ~0.1% SP cells were found in the LAPC9 tumor and these SP cells demonstrate 100- to 1,000-fold higher tumorigenicity than the corresponding non-SP cells. SP tumor cells also express high levels of several 'stemness' genes such as β-catenin and BMI-1 *(30)*. A recent study shows that SP is detected in both benign and malignant prostate tissues and that the SP cells express basal cell markers *(31)*. Whether these SP cells selected from primary human PCa samples also possess CSC properties and high tumor-initiating ability is unclear.

4.3. Sphere-Formation Assays

Normal SCs (e.g., HSCs and neural SCs) can maintain their differentiating abilities in a three-dimensional environment. Sphere-formation assays have been utilized to enrich for tumor-initiating cells in CNS tumors and melanoma (reviewed in *[10]*). The xenograft prostate tumor LAPC4 contains sphere-forming cells that, although rare, are enriched during serial passage and are highly tumorigenic in NOD/SCID mice *(10)*. These observations suggest that the sphere-initiating cells might mark tumor-initiating, putative CSCs.

5. CSCs AND METASTASIS

Nearly 85% of the advanced PCa metastasizes preferentially to the bone and rarely to some other organs such as kidney and spleen. The organotropism of metastatic lesions indicates that metastasis is not merely a random or anatomical process *(32,33)*. The 'clonal dominance and clonal selection' model proposes that metastasis is driven by genetic mutations during late stages of tumorigenesis and only tumor cell clones that have attained the 'right' combination of mutations hold the growth advantage and metastasis ability (reviewed in *[9]*). This model, however, contradicts studies by Fidler and Kripke of 30 years ago showing that metastatic variants pre-exist in primary tumor *(34)*, a contention that has gained support by more recent gene expression profiling studies *(35)*. In principle, these two models of metastasis may not

necessarily be mutually exclusive. Microarray analyses coupled with functional assays have also identified organ-specific metastasis signatures and linked these signatures to the poor prognosis of patients *(35–37)*. Gene profiling studies in PCa have identified an *11* 'stemness' gene signature in highly metastatic PCa cells, which seems to be able to predict the poor outcome in PCa patients *(37)*. Taken together, these studies strongly suggest that CSCs may be involved in tumor metastasis and several pieces of evidence support this suggestion. *Firstly*, eight out of nine breast cancer specimens from which the breast CSC were first reported were from metastasis *(4)*. *Secondly*, several commonly used CSC markers, in particular, CD44 and CXCR4, are well-established mediators and regulators of metastasis. *Thirdly*, metastasis could be derived from a single cell and only SCs with intrinsic self-renewal ability could reinitiate the clonal growth *(38)*. *Fourthly*, dormancy and drug resistance have long been recognized as traits of metastases (especially in metastatic breast and prostate tumor cells) and only the properties of CSCs can explain the two traits of the metastatic lesions *(3)*. *Finally*, CSCs might be the only cells in the tumor that could regenerate the heterogeneity observed in metastatic cancers *(39)*.

6. POTENTIAL ROLES OF CSCs IN PCa METASTASIS AND THERAPEUTIC IMPLICATIONS

How might CSCs initiate metastasis? Breast cancer researchers have speculated that oncogenic mutations cause breast SCs to transform into breast CSCs and only these CSCs, called metastatic CSCs (mCSCs), can metastasize to different organs with different intrinsic tissue-specific gene signatures *(1)*. By contrast, if the mutations occur in differentiated or progenitor cells, these mutated cells can only develop into a nonmetastatic tumor with good prognosis. Interestingly, a recent study reveals that tumor cells recruit BMDCs to form premetastasis niche before cancer cells' arrival suggesting that the 'premetastasis niche' might play a critical role in initiating metastasis *(40)*. There is also some speculation that mCSCs might interact with BMDCs by secreting extracellular matrix protein osteopontin (Opn), which aids in recruiting BMDCs and thus facilitates metastasis formation *(40,41)*.

In PCa, Tu et al. proposed a SC-origin-of-metastasis model *(11)*. Using xenograft models, we provided the first piece of experimental evidence that the $CD44^+$ PCa cell population, which possesses intrinsic CSC properties, is also enriched in metastatic PCa cells *(18)*. In fact, the $CD44^+$ PCa cells are the only cells that show metastatic capacity when orthotopically implanted in mouse prostate whereas hundreds of thousands $CD44^-$ PCa cells fail to manifest metastatic potential *(18)*. Importantly, most of the $CD44^+$ PCa cells are AR^- *(10,18)*, suggesting that prostate CSCs endowed with an intrinsic ability to metastasize are undifferentiated cells, consistent with the concept that CSCs possess the ability to undergo multilineage differentiation and re-create the heterogeneity of the original tumors.

One significant clinical implication for CSC model in PCa therapy is its prediction that the current mainstay therapy for advanced PCa, i.e., hormonal ablation (or androgen blockage) may eventually fail because, as discussed above, prostate CSCs may be mostly AR^-. Therefore, although androgen deprivation may eradicate the majority of AR^+/PSA^+, differentiated PCa cells, the AR^- CSCs may survive deprivation and re-emerge to generate a more aggressive tumor (i.e., a tumor that is less responsive to androgen deprivation due to the expansion of AR^-, undifferentiated PCa cells). Furthermore, the undifferentiated nature of prostate CSCs may help predict potential metastatic diseases in patients with undetectable or low serum PSA levels *(42)*. PSA is usually considered the most sensitive marker for PCa progression and metastasis clinically; accordingly, patients lacking PSA production are often treated as disease-free *(43)* However, the existence of PSA^-/AR^- CSCs may suggest that patients without obvious clinical PSA

spikes may need be treated carefully and require different surveillance strategies. Otherwise, these patients may develop malignant tumors later with highly metastatic propensity. In addition, by measuring CSC marker(s), we may find new ways to detect aggressive PCa early. We may also use the prostate CSC model to explore new therapeutic targets for both primary tumors and metastasis lesions. For example, if we can find some strategies to specially eliminate the $CD44^+\alpha2\beta1^-$ population in the tumor, we might eradicate the primary tumor and also prevent metastasis. On the other hand, targeting multiple pathways related to SC properties such as self-renewal, drug resistance, homing, migration, interaction with microenvironment, and hypoxia, and targeting multiple key players in these pathways such as Wnt/β-catenin, BMI-1, Opn, CXCR4, and ABCG2, might hold the greatest promise for tumor therapy. Gene expression signature might also be used to predict the metastasis potential and identify new therapeutic targets for metastasis. By isolating CSC population(s) and comparing gene expression between CSCs and normal/benign prostate tissues, we might identify CSC-specific and metastasis-specific gene signatures that are important for primary tumor growth and metastasis. It will also lead to better understanding of the mechanisms regulating these fundamental pathological processes. In conclusion, deepened knowledge on the prostate CSCs will lead to more effective intervention strategies for PCa and its metastasis.

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XI Genomic Signatures of Cancer: Basis for Selective Adjuvant Therapy

40 Estrogen Receptor-Positive Breast Cancer: Traditional Prognostics, Molecular Pathology: A New Breast Cancer Taxonomy and 21st Century Personalized Prognostic and Predictive Assays

Frederick L. Baehner, MD

Contents

ABSTRACT

Personalized Medicine, by the utilization of powerful gene expression technologies, is rapidly revolutionizing the clinical management of treatment decisions in patients with breast cancer. In addition to powerful traditional clinicopathologic metrics the new technologies offer the personalized assessment of individual patient tumor samples. By accurately quantitating gene expression and integrating these precise measurements into powerful biostatistical models, the risk of distant recurrence and the potential benefit of chemotherapy may be assessed for individual patients and their health-care providers. This personalized medical information allows for actionable, individualized treatment decisions. In addition to a detailed review of the traditional clinicopathologic metrics, this chapter will review current molecular assessment technologies as well as review landmark trials that use these tools in a prespecified manner in

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clinical validation trials and that demonstrate their clinical utility in the management of individual patient treatment decisions.

Key Words: gene expression profile; QRT-PCR (quantitative real-time polymerase chain reaction); breast carcinoma; basal type breast cancer; HER2 type breast cancer; luminal type breast cancer; prognostic; predictive

> No classification is perfect nor is it likely that it ever will be . . . All classifications depend on our knowledge of the pathology and histogenesis of the tumors being classified and, since this knowledge is far from perfect or complete, no classification can be other than a reasonable working compromise
>
> Dr. John Azzopardi (2).

1. INTRODUCTION

1.1. The Dawn of Molecular Pathology

Molecular pathology was born in 1960 when Nowell et al. described a minute chromosome in human chronic myeloid leukemia (CML). In 1973 it was recognized as the Philadelphia Chromosome which is characterized by the t(9;22) translocation *(39)*. With continued technologic advancement, the molecular pathology of CML was further refined and found to be due to unregulated signal transduction by the BCR-ABL tyrosine kinase. This constitutively activated kinase results from the fusion of the *BCR* (breakpoint cluster region) gene on chromosome 22 to the *ABL* (Ableson leukemia virus) gene on chromosome 9. This is the sine qua non of CML. The Philadelphia Chromosome also provides prognostic and predictive information. In regards to the latter, it predicts response to Imatinib, an orally available ABL kinase inhibitor that can induce hematologic and cytogenetic remission in all stages of CML.

1.2. Breast Carcinoma

Similar advancements in prognostic and predictive factors as well as tailored treatments have occurred in breast cancer. This began at the onset of the 20th century with recognition of the prognostic significance of tumor grading and histologic subtypes and it culminated at the end of the century with the identification of hormone (estrogen and progesterone receptors) and growth (HER2) receptors as therapeutic targets. It is interesting to note, that methods for the semiquantitative assessment of breast tumor grade were described before a full classification of histologic types was established *(27,51)*. Today, the prognostic factors required by College of American Pathologists include, lymph node status, tumor size, tumor grade, histologic type, hormone receptor status, skin/chest wall involvement, and lymphovascular invasion (optional) *(20)*.

2. TRADITIONAL PROGNOSTIC AND PREDICTIVE FACTORS

2.1. Introduction

Breast carcinoma is the second leading cause of cancer death in women. The pathologist's role in patient management is to correctly diagnose and classify the tumor and then provide key prognostic information to the clinician and patient: tumor type, tumor size, tumor grade, margin status, presence of lymphovascular invasion, and axillary lymph node status. This information helps determine whether the tumor will recur, either locally or distantly, and whether the patient may benefit from hormonal and/or adjuvant chemotherapy. Unfortunately, for individual patients, these metrics do not precisely quantitate the risk of distant recurrence or chemotherapy benefit. Breast carcinoma is a

heterogeneous disease, with equally heterogeneous outcomes. Invasive duct carcinoma, not otherwise specified, is the largest group of malignant mammary tumors, constituting 65–80% of mammary carcinomas, but within this cohort, patient outcome is extremely varied. Heimann recently articulated the dilemma as follows *(29)*:

> ...Required of tumors is the development of critical phenotypic attributes: growth, invasion, metastagenicity, and angiogenesis...Recognizing tumor heterogeneity emphasizes the need to determine an individual tumor's place in the evolutionary spectrum. This may be accomplished using clinical feature such as size, nuclear grade, patient age, as well as by examining angiogenesis, metastatic capacity, and proliferation. Identification of the extent of tumor progression with regard to these major tumor phenotypes should allow individual therapy to be fashioned for each patient.

The future of personalized medicine and the development of prognostic and predictive tools will require, in addition to the "tried and true" traditional clinicopathologic metrics, the discovery of genes predictive of distant recurrence and chemotherapy benefit.

2.2. Tumor Stage: Axillary Lymph Nodes

The presence of metastatic disease within the axillary lymph nodes is still the single most powerful prognostic factor for risk assessment in primary breast cancer. It has been noted in numerous studies that there is a direct relationship between the number of positive lymph nodes and clinical outcome (Fig. 1).

To date, there have been no genes or other molecular factors that have been prospectively validated to identify a priori patients at risk of being node positive; and, as such, sentinel lymph node biopsy (SLN) continues to be the standard of care.

2.3. Isolated Tumor Cells: pN0i+

By definition isolated tumor cells (ITCs) are tumor cell clusters that do not measure larger than 0.2 mm. No other diagnostic guidelines have been published and there is histologic ambiguity in this definition. Furthermore, the clinical significance of such clusters has not been determined. Connolly recently pointed out the obvious diagnostic questions that pathologists face when confronted with ITCs: what about spaces between clusters of malignant cells, how are

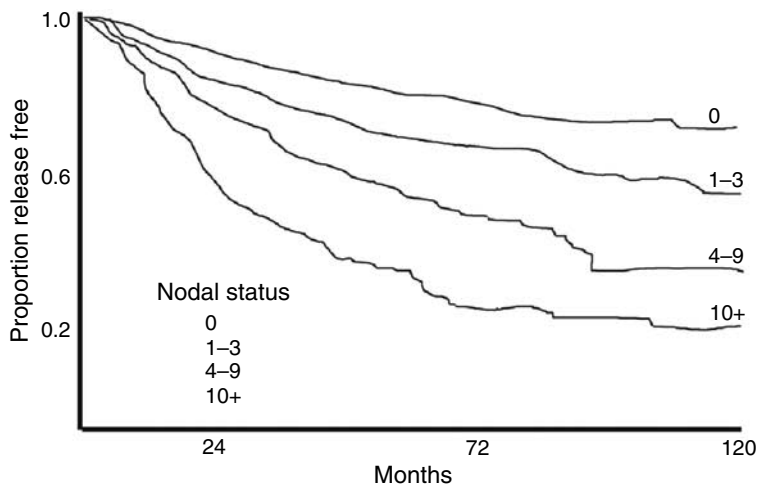

Fig. 1. Relapse-free survival by number of axillary lymph nodes ($n = 2{,}873$ patients, median follow-up of 37 months) (from Chang and Hilsenbeck as presented in the *AJCC Staging Manual*, 6th edition *[7]*).

these small clusters with intervening normal lymphoid tissue measured, how close do the deposits need to be to sum their measurements and what about diffuse involvement of the lymph node by ITCs, as is often seen with infiltrating lobular carcinoma. In this latter case, while these are truly ITCs, it is recommended to classify such a node as positive (pN1a), based on the number of tumor cells *(9)*.

2.4. AJCC Cancer Staging Manual

The 6th edition of the *AJCC Staging Manual* updates the definitions of pN0(i+) and pN0(i–). The updates state that i+ or i– refers to the presence or absence of ITCs detected by *any* morphologic technique, including hematoxylin–eosin staining and immunohistochemistry (IHC).

- pN0(i–) is: no regional lymph node metastasis histologically, including negative morphologic findings for ITCs (by any morphologic technique, including hematoxylin–eosin and IHC).
- pN0(i+) is: no regional lymph node metastasis histologically, but positive morphologic findings for ITCs (any morphologic technique, including hematoxylin–eosin and IHC), and no ITC cluster greater than 0.2 mm.

2.5. Micrometastases: pN1mi

Tumor deposits greater than 0.2 mm but not greater than 2.0 mm in largest dimension are termed micrometastases (pN1mi). Cases in which only micrometastases are detected (none greater than 2.0 mm) are classified pN1mi. The prognostic significance of micrometastases is unclear *(49,10,11,52)*. Retrospective studies have reported decreases in disease-free survival (DFS) ranging from 10 to 22% in some subgroups of patients where nodal micrometastatic disease was detected by immunohistochemistry. Most recently, at the Breast Cancer: Current Controversies and New Horizons Meeting, it was reported that patients with micrometastases *after chemotherapy* fared as poorly as patients with macrometastases; whereas, in patients with micrometastases *prior to treatment*, the prognostic significance has yet to be determined. Studies are ongoing to better define the optimal treatment for this subgroup of patients.

2.6. Tumor Size

After nodal status, tumor size is one of the most consistently prognostic factors for predicting risk of distant relapse, especially in lymph node-negative patients as illustrated from one data set shown below (Fig. 2). Given increased mammographic surveillance, many tumors are noted earlier and this impact on outcome is unclear. Are more malignant tumors being caught earlier, at a smaller size? Does this "stage migration" bias the historical size staging data? The varied outcomes of similarly staged patients by size is most consistent with breast cancer not being a homogeneous disease, but rather a spectrum of disease states, with varying capacities for growth and metastasis.

After >15 years, untreated lymph node-negative, estrogen receptor (ER)–positive (ER+) patients with tumors less than 2.0 cm still have a risk of distant recurrence of 25%. The Memorial Sloan Kettering Cancer Center group has advocated a 12% risk of relapse for tumors less than 1 cm *(48)*. Even tumors less than 1.0 cm have a 10% chance of distant metastasis and such patients may benefit from adjuvant chemotherapy *(17)*. Thus systemic therapy is now considered even for patients with small node-negative breast cancer.

2.7. Tumor Grade

Semiquantitative assessment of breast tumor grade was described before a full histologic system of classification was established. Tumor grading was contentious over 50 years ago when Willis, an influential figure in pathology, stated that attempts at precise numerical

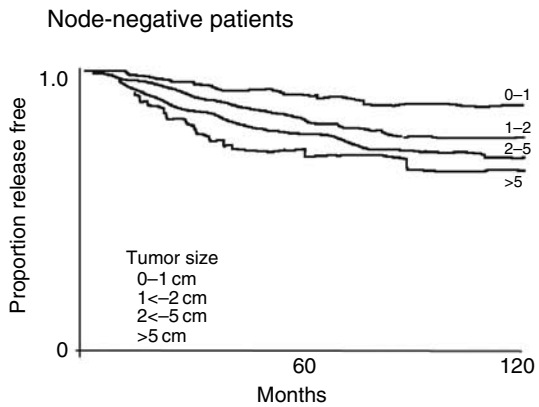

Fig. 2. Relapse-free survival by tumor size for node-negative patients ($n = 1{,}613$ node-negative patients, median follow-up of 43 months) (from Chang and Hilsenbeck as presented in the *AJCC Staging Manual*, 6th edition *[7,30]*).

histological grading of tumors were arbitrary, unscientific and wasted effort *(62)*. Today, the standard method is the Nottingham Combined Histologic Grade (NCHG) or Elston–Ellis modified SBR (mSBR) (Table 1) *(12,36)*. Although some studies show poor reproducibility and lack of agreement among different observers *(25)*, when performed by experienced pathologists histologic grade has been shown to correlate with clinical outcome as is illustrated from one data set shown below (Fig. 3) *(5,15,18,19,21)*. According to the College of American Pathologists, all invasive breast cancers, except medullary carcinoma, should be graded.

Perhaps the most poorly reproducible component is the assessment of nuclear pleomorphism and as such deserves a word. According to Elston and Ellis:

> ...to introduce a degree of objectivity we have suggested that the size and shape of normal epithelial cells present in breast tissue adjacent to the tumor should be used as a reference point...When the tumor nuclei are small, with little increase or variation in size compared with normal nuclei and have regular outlines and uniformity of nuclear chromatin, one point is appropriate. A score of 2 points is given when the nuclei are larger than normal, have more open vesicular nuclei with visible, usually single, nucleoli and there is a moderate variation in

Table 1
Summary of Semiquantitative Method for Assessing Histological Grade In Breast Carcinoma *(16)*

Feature	*Score*
Tubule formation	
Majority of tumor (>75%)	1
Moderate degree (10–75%)	2
Little or none (<10%)	3
Nuclear pleomorphism	
Small, regular nuclei	1
Moderate increase in size and variability	2
Marked variation	3
Mitotic counts	
Dependent on microscopic field	1–3

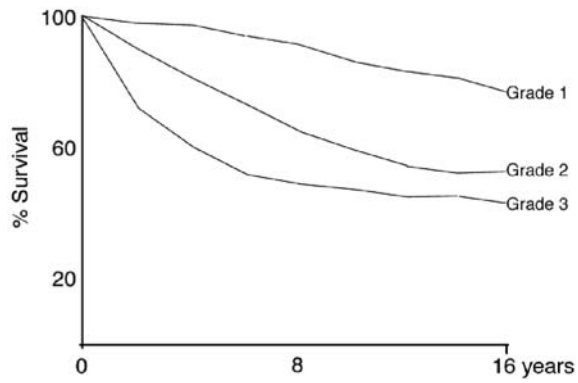

Fig. 3. Correlation between histologic grade and overall survival in 2,005 patients with primary operable carcinoma of the breast from Elston & Ellis's *The Breast (16)*.

size and shape. A marked variation in size and shape, especially when very large and bizarre nuclei are present, scores 3 points; nuclei are vesicular with prominent enlarged and often multiple nucleoli *(16)*.

2.8. Histologic Subtypes

Pure papillary, tubular, and mucinous morphologies identify a small subset of patients with a better prognosis than infiltrating ductal carcinoma. However, the majority of tumors (80–90%) are ductal carcinomas of no special type (NOS) and the risk of recurrence with ductal carcinoma, NOS is quite variable *(16)*.

2.9. Hormone Receptors: An Introduction

More than one hundred years ago Beatson noted that an oophorectomy increased survival in some patients with advanced breast carcinoma *(3)*. Almost 40 years ago researchers first noted that radiolabeled estrogens were preferentially concentrated in human breast cancers and that many breast cancers are dependent on estrogen and/or progesterone for growth *(31)*. The receptors for estrogen and progesterone (ER/PR) are highly expressed in the majority malignant breast carcinomas (~75%). ER exerts effects through:

1. Classical ligand-dependent pathway in which the ER complex regulates gene transcription through its interaction with estrogen response element (ERE) consensus DNA sequences.
2. Ligand-independent pathway in which growth factors and their tyrosine kinase receptors may activate ER and increase the expression of ER target genes in the absence of estrogen.
3. DNA binding-independent pathway in which induction of gene regulation by ER complexes is through interactions with no ERE-like promoter elements such as AP1, SP1, and CREs.
4. Cell surface (nongenomic) signaling in which estrogen activates a putative membrane-associated binding site that generates rapid tissue responses.

However, the details of the estrogen effect on downstream gene targets, the role of cofactors, and crosstalk between other signaling pathways, at least until the past few years, was largely unknown.

What is known is that all four mechanisms contribute to ER proliferative and survival effects. As the ER has become the target of hormonal therapy, ER status is now recognized as a strong predictive factor. PR status has been variously reported as prognostic and/or predictive *(35)*. Of great significance is that patients with hormone receptor-positive disease do not have a uniform response to endocrine therapy.

2.9.1. The Estrogen and Progesterone Receptors

There are two ERs, ERα (chromosome 6) and ERβ (chromosome 14) and two PRs, PRA and PRB. They share a common structural and functional organization: their functional domains have been designated A–F (ERα, Fig. 4). The ERα gene is complex, 140 kb with eight exons and it has 595 amino acids with a central DNA-binding domain (DBD) and a carboxyl-terminal, hormone-binding domain (HBD). Binding of estrogen to ER activates the receptor with coincident dissociation of chaperonin proteins, i.e., heat shock protein 90. Hormone-bound ER then dimerizes and binds to EREs in the promoter of estrogen-responsive genes. Through binding the DNA, ER influences expression of estrogen-responsive genes such as PR. There are two distinct activation domains: (1) a hormone independent domain in exon 1 and 2 (AF-1) and (2) a hormone dependent, carboxyl-terminal activation domain is contained in portions of exon 4 through 8 (AF-2). AF-1 and AF-2 activate transcription independently and/or synergistically. Both are needed for maximal ER transcriptional activity. Tamoxifen inhibits AF-2 activation and thereby ER activity but tamoxifen does not prevent activation of AF-1. Additionally, when AF-2 is not required and AF-1 is sufficient for ERα activity, then tamoxifen can function as a partial agonist *(58)*.

2.10. ER Negativity

Tumors lacking the ER have been postulated to be negative due to genomic deletion or rearrangement, gene methylation, and lack of gene transcription. Most ER–negative (ER–) tumors show little ER mRNAs *(47)*. Variant ERα isoforms due to exon deletions have been described. Exon 5 deletion, involves the HBD domain and results in constitutive, hormone-independent activity. Missense mutations have been described but appear rare. Those reportedly of significance include tyrosine 537 asparagine (causing constitutive activation).

2.10.1. Estrogen Receptor β

On the one hand, ERβ has been associated with clinical tamoxifen resistance and is thought to enhance tumor aggressiveness; while, on the other hand, most studies have found it to be a marker of good prognosis. Critics contend that the studies have not been powered appropriately and larger studies appear to be required. ERβ is generally coexpressed with ERα (76% ERα+/β, 14% ERα+/ERβ–, 15% ERα–/ERβ+) *(24)*.

2.10.2. Methods of Measuring Hormone Receptors

Most of the data on the clinical utility of ER content have been generated using biochemical ligand-binding assays (LBAs), such as the dextran-coated charcoal assay (DCCA). Since the first report of ERs independent prognostic significance almost two decades ago, the assessment of ER status by DCCA has been validated repeatedly and is generally regarded as the standard by

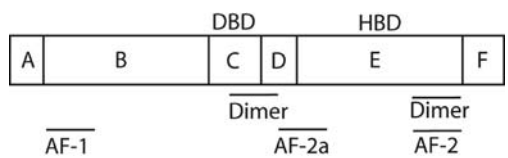

Fig. 4. A diagram of ERα functional domains labeled A through F. The central DNA-binding domain (DBD) and hormone-binding domain (HBD). Regions important for dimerization (dimer) and transactivation functions (AF-1, AF-2a, AF-2), respectively. Region A/B (AF-1) is key for hormone-independent ER transcription, region F is important for modulation of ER activity (AF-2).

which other methods are assessed *(34)*. Currently, the validated method of choice is immunohistochemistry, performed on fixed, paraffin embedded tissue *(45)*. An emerging method is by QRT-PCR to measure mRNA levels of ER and PR *(41)*.

2.10.3. Methods of Measuring Hormone Receptors: the Ligand-Binding Method

The ligand-binding method (DCC) measures radiolabeled steroid (ligand) bound to the homogenized breast tissue. Using a standardized Scatchard plot, total concentration of receptor protein in the cytosol is obtained and expressed as femtomoles of receptor protein per milligram of total cytosol protein. The most common cutoff for considering a tumor positive was >3.0 fmol/mg (others have used >10 fmol/mg). Specimens needed to be immediately frozen and shipped on dry ice to prevent protein degradation. The tumor needed to be reviewed so that representative portions, from 0.5 to 1.0 cm in diameter could be chosen. Variability in results was caused by inclusion of benign tissues, tumor receptor heterogeneity, pregnancy, delayed time to tissue freezing, or tamoxifen usage. DCC was complicated, required central laboratories, and used low-level radioactive materials.

2.10.4. Methods of Measuring Hormone Receptors: Immunohistochemistry

IHC uses antibodies specifically directed against epitopes unique to each receptor protein and can be used on all fixed specimens as well as frozen sections. Correlation between DDC and IHC is high, from 80 to 90% *(1)*. PR has been compared, though less exhaustively and concordance is 70–80% *(38)*. Problematic is variability of result and as many as 10% of cases may be incorrectly classified. This may occur due to fixation differences (fixative, duration of fixation), differences in assay protocol (particularly antigen retrieval, widely thought to be the most important factor in assay variability *(46)*) and antibody clone. Additional problems include that the method is semiquantitative and interpretation is subjective. Attempts to automate staining and the use of image analysis systems, such as the ACIS II (DakoCytomation), are steps toward greater objectivity in quantitation of hormone receptor expression.

2.11. Protocol: Estrogen Receptor

Consistent ER results have been repeatedly obtained and reported from tissue sections fixed for 6–8 h in 10% neutral buffered formalin *(26)*. The debate over the best monoclonal antibody continues. The most widely used antibodies are 6F11 and 1D5, both mouse monoclonal antibodies and SP1, a rabbit monoclonal with reported increased affinity. 1D5 is perhaps the most widely used and clinically validated antibody *(28)*. Recently, there has been concern that ER assessment using the 1D5 antibody misclassifies ~5–10% of breast cancers as ER– *(6)*. Cheang and colleagues contend that SP1 is more sensitive, better predicts tamoxifen response, and is better correlated with the LBA (Fig. 5 and Color Plate 29). The new ER/PR pharmDX assay is a Federal and Drug Administration (FDA) 510(k) cleared assay and includes both 1D5 and a second ER antibody, ER-2-123.

2.12. Clinical Validation: Estrogen Receptor as a Continuous Variable

To identify a clinically meaningful cut point for defining ER+ tumors, Allred et al. developed and used an eponymous scoring system that measures both the proportion of cells staining (5 pts, 100%; 4 pts 66%; 3 pts, 33%, 2 pts, 10%, 1 pt, 1%; 0 pt, 0) and their intensity (3 pts, strong; 2 pts, intermediate; 1 pt, weak) *(28)*. The proportion of cells staining was added to the intensity of staining and results in a score from 0 to 8. Using DFS curves, tumors were defined as ER+ if their total IHC score was greater than 2 and ER– if their score was 0–2. Note that an Allred score of 3, the lowest

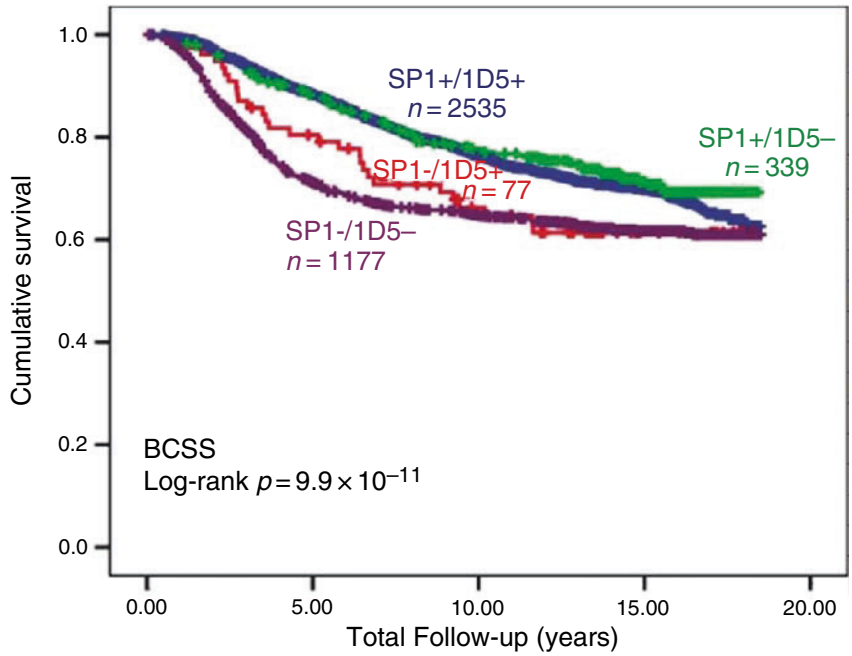

Fig. 5. Comparison of ER antibodies SP1 and 1D5. The SP1 + /1D5– cases have cumulative survival at 10 years similar to those of SP1 + /1D5 + tumors *(6)*. (*see* Color Plate 29)

possible positive score, corresponds to as few as 1% intermediately staining tumor cells to 10% weakly staining tumor cells. For patients receiving no systemic adjuvant therapy ($n = 701$), ER status was only a weakly prognostic factor. For patients who received adjuvant endocrine therapy, either alone ($n = 517$) or in combination with chemotherapy ($n = 260$), ER status was a highly significant predictive marker of DFS. For these latter two groups combined ($n = 777$), the best cut point (IHC score > 2) was highly significant ($p > 0.0001$) (Fig. 6). It is not yet known whether other laboratories performing this assay would obtain similar results, or a similar cutoff point.

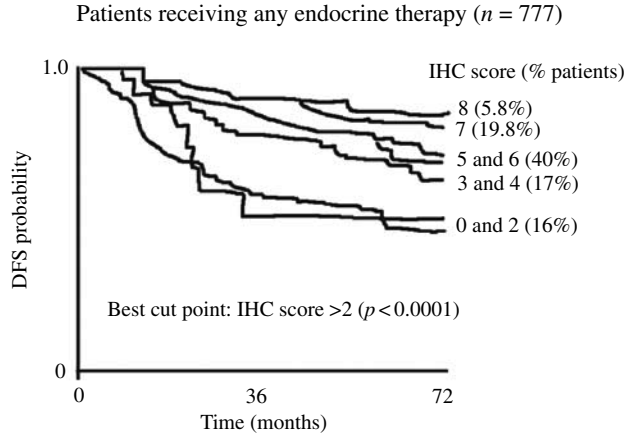

Fig. 6. Disease-free survival (DFS) curves for all possible IHC scores within the different treatment groups. For patients who received adjuvant endocrine therapy, either alone (n = 517) or in combination with chemotherapy (n = 260), ER status was a highly significant predictor of DFS. For these latter two groups combined (n = 777), the best cut point (IHC score > 2) was highly significant ($p > 0.0001$). On the basis of these results, tumors were defined as ER + if their total IHC score was greater than 2 and ER– if their score was 0 or 2 *(28)*.

2.13. Clinical Validation: Progesterone Receptor

PR has been considered a surrogate marker of ER activity in breast cancer *(38)*. Its utility in predicting clinical outcome has been established using biochemical assays. Allred et al. validated an immunohistochemical assay for PR in breast cancer using the monoclonal antibody 1294, the slides were scored microscopically using the Allred score on a scale of 0–8. The assay was compared to LBA in 1,235 breast cancers, and a subset ($n = 362$) that received only hormonal therapy was used to define a cutoff for PR positive. Clinical utility was validated in an independent set of samples ($n = 423$) from a clinical trial randomizing premenopausal breast cancer patients to tamoxifen + oophorectomy versus observation following surgery. A cutoff of Allred score > 2 (corresponding to $> 1\%$ positive cells) dichotomized patients with significantly better or worse clinical outcome ($p = 0.0014$). PR by immunohistochemistry provided significantly better results than PR by LBA in predicting clinical outcome. In the clinical trial, a positive result in univariate analyses was associated with significantly improved disease-free and overall survival (OS) both in untreated and hormonally treated patients. Positive progesterone receptor remained significant for improved disease-free and OS in multivariate analyses including the standard variables of tumor size, nodal status, treatment, histological grade, and HER2 status (PR remained a strongly prognostic factor). ER and PR were noted to be codependent variables and progesterone receptor was a weaker predictor of response to endocrine therapy than ER when both were included in multivariate analysis (PR is only weakly predictive of hormone therapy).

Debates: Hormone Receptor Testing a Continuous Variable or Dichotomous?

Recently, Collins et al. analyzed 825 cases using the Allred score (Fig. 7). They noted that in 817 cases (99%), tumor cells showed either complete absence (score 0) or strong immunostaining of 70% or more of cells (score 7 or 8). They concluded that with the immunohistochemical method used in their laboratory, ER staining is essentially bimodal. The overwhelming majority of breast cancers were either completely ER– or unambiguously ER+, and cases with weak ER immunostaining were rare. The debate over whether ER expression is continuous or dichotomous continues.

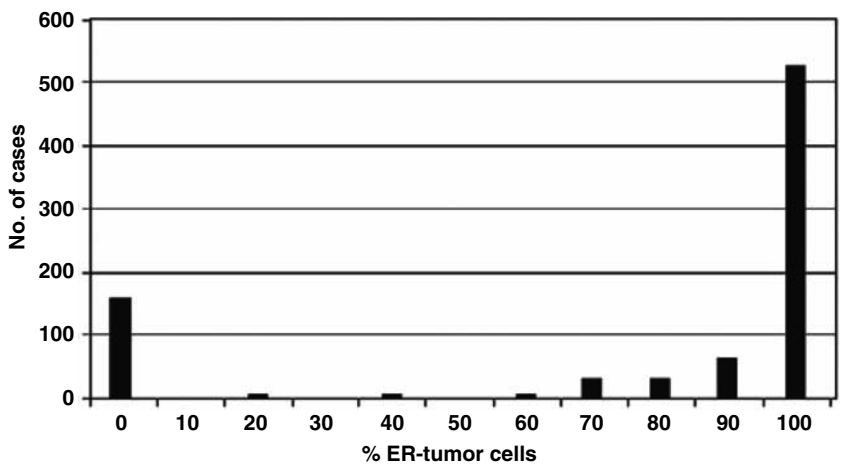

Fig. 7. Frequency distribution of the percentage of cells showing "bimodal" or dichotomous nuclear staining for estrogen receptor among 825 primary breast cancers *(8)*.

2.14. Endocrine Therapy

Although the levels of ER are weakly prognostic with regards to breast cancer recurrence and OS, the true clinical utility of ER is that it is a as powerful, predictive marker for benefit of endocrine therapy. The Early Breast Cancer Trialists' Collaborative Group data have confirmed the survival benefit for the average patient with early-stage disease (especially younger women) by tamoxifen for any patient with ER + disease regardless of age *(63)*. Aromatase inhibitors have been show in the Arimidex Versus Tamoxifen Alone or in Combination (ATAC) trial and the Breast International Groups (BIG) 1–98 trial to improve disease-free survival (DFS) over tamoxifen, but thus far none have demonstrated a significant improvement in OS.

2.14.1. Tamoxifen-Resistance in ER + Breast Cancer

The expression of ER is the most important factor in predicting endocrine response and classification of ER + tumors is made by detecting ERα. However, ER expression alone does not accurately predict response to endocrine therapy as response to tamoxifen in ER + patients is less than 50%. ER status/expression does remain positive in most tumors which initially are tamoxifen responsive but then develop resistance. Interestingly, over two thirds of patients who develop resistance will respond upon changing endocrine therapies.

There are many theories about the cause(s) of tamoxifen resistance. Perhaps most likely is activation of AP-1-response elements that regulate genes involved in cell proliferation, motility, and apoptosis. Enhanced AP-1 activation has been associated with tamoxifen resistance *(32)*. Recently, *nongenomic* mechanisms for ER action have been postulated as alternative mechanisms for ER + endocrine resistance. ER can cause rapid tyrosine phosphorylation of SHC. A transient activation of extracellular signal-regulated kinases ERK1 and ERK2 is mediated by ER. This may result in different cell survival and proliferative signals via the AKT and mitogen-activated protein kinase (MAPK) pathways (Fig. 8) *(53)*. Additionally, tamoxifen may exert agonist effects via the interaction of membrane ER with growth factor receptor signaling (epidermal growth factor receptor [EGFR] or HER2). Finally, "crosstalk" may occur in ER + tumors with HER2 amplification as activation of MAPKs, ERK1, and ERK2 by overexpressed HER2 may result in phosphorylation of ER, resulting in tumor stimulation rather than inhibition *(33,55,56,63)*.

2.14.2. Acquired Endocrine Resistance

EGFR and HER2 seem to become selectively upregulated in breast cancer cells that acquire resistance to tamoxifen during prolonged exposure. Overexpression of HER2, amplified in about 10% of ER + breast cancers, results in loss of tamoxifen's ER antagonist activity and the acquisition of tamoxifen-stimulated growth. Tamoxifen, like estrogen, may activate HER2 via the membrane functions of ER, which, in turn, phosphorylate both ER and AIB1 (SRC3), an important ER coactivator.

The cumulative data from clinical studies support the hypothesis that overexpression of HER2, EGFR, and high levels of phosphorylated AKT or ERK, appear to contribute to tamoxifen resistance in some patients. Aromatase inhibitors (estrogen deprivation), on the other hand, may be more effective in such tumors since ligand deprivation would shut off both nuclear and membrane ER activity, thereby eliminating the crosstalk generated in the presence of estrogen or tamoxifen.

2.14.3. Other Markers

Other markers of prognostic significance have been p53 mutated forms, patient age, patient menstrual/menopausal status, race, EGFR, epidermal like growth factor B-2 (HER-2/neu), Bcl-2, and plasminogen activators. These are outside this discussion.

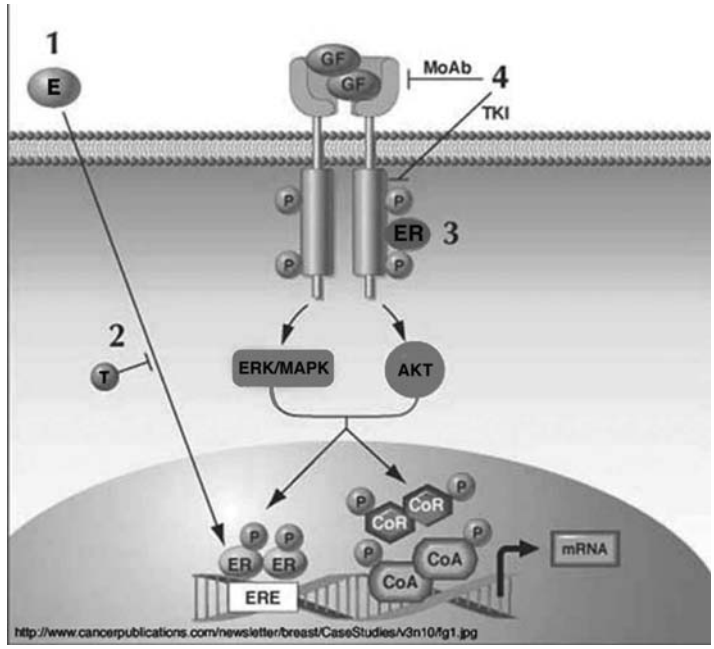

Fig. 8. Diagram of genomic and nongenomic mechanisms of estrogen receptor (ER) action and resistance. (1) Estrogen exerts its effect through ER on nuclear transcription (genomic). (2) Binding with the ER is blocked by tamoxifen. (3) Activation of EGFR/HER2, either by overexpression or by ER "crosstalk," induces phosphorylation of the ER, coactivators, and corepressors by the MAPK/Akt pathways, resulting in increased ER activity and resistance to tamoxifen. (4) Inhibiting EGFR/HER2 may restore sensitivity to tamoxifen *(50)*.

3. PERSONALIZED MEDICINE: 21ST CENTURY PROGNOSTIC AND PREDICTIVE MOLECULAR TOOLS

3.1. Roadmap

The ensuing discussions will examine new molecular methodologies and their role in defining the current breast carcinoma taxonomy and new and/or improved prognostic and predictive markers. Technology is the driving force in many of these developments and it includes QRT-PCR, gene expression microarrays, and comparative genomic hybridization (CGH). These techniques will be introduced below. CGH involves examining tumor DNA, looking for large genomic gains and deletions. Expression microarrays were first described in the mid-1990s as a way to examine the expression (mRNA levels) of thousands of genes simultaneously *(37)*. QRT-PCR is a robust, precise, sensitive, and reproducible way to quantify tumor mRNA. The study of DNA is referred to as *genomics* while the study of mRNA is referred to as *functional genomics*.

3.2. Technology Introduction

3.2.1. Microarray Analysis: What Is It and Why Is It Valuable?

As morphologists, pathologists are experts at examining fixed paraffin sections that have been stained with hematoxylin–eosin. By identifying mitotic figures the pathologist is able to assess the proliferative activity of a cell. Immunohistochemical stains for the Ki-67 protein allow for semiquantitative monitoring of the growth fraction of normal and neoplastic cells. DNA microarray analyses measure quantities of individual messenger RNA, that is, *gene expression*. Using this method, a snap shot of all the proliferation genes that a cell is transcribing can be examined

and quantified at once. Indeed, the entire genome maybe surveyed at once. DNA microarray analysis was first used to compare different tumors to identify genes and functional gene groups that differed in relative expression, i.e., the ER and associated genes, as they differ between ER + and ER– tumors. Such comparisons in *gene expression* have enabled identification of genes relevant to cancer progression *(13)*, to new tumor subclasses *(43)*, and to the identification of biomarkers associated with disease.

3.3. DNA Expression Microarrays: "Expression Arrays"

3.3.1. Functional Genomics

Gene microarray analysis allows for the quantification of thousands of unique messenger RNA (mRNA), obtained from fresh frozen tissue samples, on a single slide or chip. From an individual tumor, levels of mRNA can be detected, quantitated, and compared among multiple tumor samples. This method is excellent for assessing correlations in gene expression, i.e., expression patterns, obtained from thousands of genes which can be assessed on a single array.

DNA microarrays are available commercially and all are based on hybridization of nucleic acid strands but there are differences between platforms. An important distinction is the length of the probe. Probes are gene-specific and represent part of a gene. Microarrays may be classified as either: cDNA arrays with probes up to a thousand base pairs (mer) or oligonucleotide arrays using short 25–30mers or long oligonucleotide (60–70mer) probes. The probes can be either contact-spotted, ink-jet deposited, or directly synthesized on the substrate. An array approximates the size of a glass microscope slide and it typically has thousands of individual genes represented on its surface, each gene represented multiple times (different segments of the gene at each site, respectively). Thousands of copies of the probe are printed or synthesized ("*arrayed*") at each site on an inert substrate (such as a glass slide or cartridge) in spatially specific locations.

3.4. Gene Arrays: Technical Comments (14)

For the most part, DNA expression arrays require fresh frozen tissue (new assay, DASL: cDNA-mediated annealing, selection, extension, and ligation appears to be a promising exception) *(4)*. The sensitivities of the various platforms range from being able to detect from 1 to 10 copies per cell. They measure relative or absolute transcript concentrations of genes above the sensitivity level of the microarray (rendering ~1/2 of the transcriptome beyond reach of arrays). They have modest reproducibilities (the best reported for Affymetrix, Agilent, and Codelink are 0.9). Cross-platform correlations can be difficult due to only moderate correlation coefficients.

3.5. Gene Expression Arrays: Methodology

- Typically RNA is extracted from fresh frozen tissue and reverse transcribed into cDNA.
  - Some newer assays are available for research purposes using fixed paraffin embedded tissues.
- cDNA is labeled with a detectable fluorescent dye, the efficiency of this labeling step is crucial for assay precision and reproducibility.
- The cDNA is placed on the array and allowed to hybridize to the arrays.
  - Individual molecules hybridize to complementary *gene-specific* probes on the array.
- Images are captured with the use of confocal laser scanning.

- The relative fluorescence intensity of each gene-specific probe is a measure of the level of expression of the particular gene.
  - The greater the degree of hybridization, the more intense the signal, implying a higher relative level of expression (more copies binding to the array).
- Comparisons from different laboratories and across various types of microarray may differ significantly! *(14)*
- After collection, the data are normalized for comparison between the different assays.
  - Normalization compensates for differences in labeling, hybridization, and detection methods.
  - Normalization and filtering transformations must be carefully applied due to effects on the results.
- The data are then filtered by objective criteria or statistical analyses to select expression levels that correlate with particular groups of samples.
- Different methods of statistical analysis applied to the same data set can produce different sets of significant genes.
  - Validation is important (confirmatory testing in a second series of patients/samples).
- Caution should be exercised in comparing data sets from different laboratories. Comparisons of published lists of genes can produce discordant results, because they rarely take into account the differences in the methods of analysis of the data.

3.6. Comparative Genomic Hybridization: Methodology

CGH provides an overview of DNA sequence copy number changes (losses, deletions, gains, amplifications) in a tumor specimen and maps these changes on normal chromosomes (Fig. 9 and Color Plate 30). CGH is a powerful method for molecular cytogenetic analysis of tumors. CGH is based on the in situ hybridization of differentially labeled total genomic tumor DNA (red) and normal reference DNA (green) to normal human metaphase chromosomes. It may be used with fixed or fresh tissue and the fact that it works with fixed tissues explains its popularity. After hybridization and differential fluorescent staining of the bound DNAs, copy number variations among the different sequences in the tumor DNA are detected by measuring and comparing the tumor to normal fluorescence intensity ratio for each locus in the target metaphase chromosomes. Each chromosome is represented by a software package as an *ideogram* (Fig. 9). Areas of *increased* copy hybridization of tumor DNA are represented in red. Areas of *decreased* copy hybridization are represented in green.

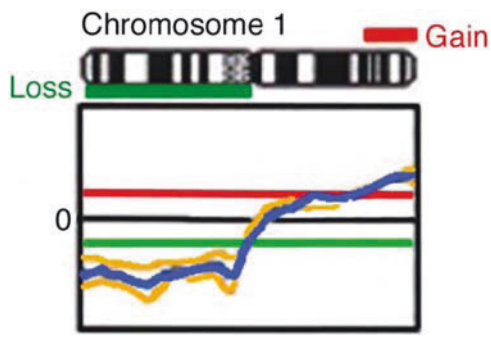

Fig. 9. This is an example of the hybridization of reference DNA (*green*) and tumor DNA (*red*) to an interphase spread of chromosomes (ready for mitosis and frozen by adding colchicine to stop spindle apparatus). Above the *red* line shows amplification of tumor gene copy and below the *green* line shows loss of gene copy number. (*see* Color Plate 30)

CGH is excellent for low resolution maps of genomic gains and losses. It is a poor method if a high-resolution map is required (sensitivity of ~50 kb).

CGH is particularly useful for analysis of DNA sequence copy number changes in common solid tumors where high-quality metaphase preparations are often difficult to grow, and where complex karyotypes with numerous markers, double minutes, and homogeneously stained chromosomal regions are common. CGH only detects changes that are present in a substantial proportion of tumor cells (i.e., clonal aberrations). It does not reveal translocations, inversions, and other aberrations that do not change copy number. Gains or losses less than 50 kb will not be identified. At present, CGH is a research tool that complements previous methods for genetic analysis. CGH will advance understanding of the genetic progression of cancer and highlight important genomic regions for further study.

3.7. Array CGH

Array-based CGH (array-CGH or a-CGH) is a modification of standard chromosomal CGH using cloned fragments of DNA spotted onto glass slide arrays as the target of hybridization, rather than normal metaphase spreads. As in conventional CGH, differentially labeled tumor and normal reference DNA are hybridized to this array, and a normalized ratio of tumor to reference intensity is calculated for every clone. This application takes advantage of the mapping information and the cloned DNA fragments generated by the human genome project. The DNA, which is spotted onto glass slides to create CGH arrays, is prepared from bacterial artificial chromosomes (BACs), which contain ~100,000 base pairs of cloned normal DNA derived from carefully mapped regions of the human genome. Current CGH arrays include BACs coding for 100 kb of DNA distributed on average every million base pairs (10% coverage, 1 megabase resolution). The next generation of CGH arrays currently being developed and validated will contain ~30,000 BACs of 100 kb each, covering 100% of the genomic sequence. Again, this is useful for fixed tissues.

3.8. Quantitative Real-Time Polymerase Chain Reaction

This method allows for the quantitation of mRNA transcripts. This characterizes the cell's gene expression at the moment the cell was fixed. It is highly sensitive, specific, precise, and reproducible. It is generally considered to be the "gold-standard" for measurement of gene expression. Figure 10 and Color Plate 31 describes one widely used QRT-PCR method, the TaqMan assay (Applied Biosystems; Foster City, CA). In brief:

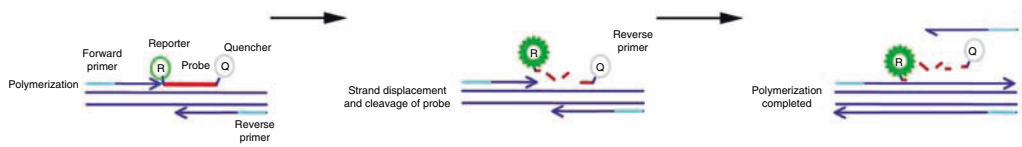

Fig. 10. QRT-PRC. The RNA is extracted, reverse transcribed and mixed with two specific primers (generally within 100 bp of each other in the case of formalin-fixed tissue) and a fluorescently labeled probe specific for thesame target. The probe has both a fluorescent reporter attached to one end, and a quencher on the other—this quenches the fluorescent signal. The primers and probe anneal to the specific target gene. Due to the 5′-exonuclease activity of the Taq polymerase, already-bound fluorescent-labeled probe is degraded while new PCR product is being synthesized. This process releases the fluorescent tag from the quencher and generates a fluorescence signal that is directly proportional to the amount of PCR product in the tube. This signal can be quantitated and the amount of mRNA determined. (*see* Color Plate 31)

1. mRNA is extracted from a fixed or fresh tissue.
2. Complementary DNA (cDNA) is made from the total RNA using oligo-dT priming, random priming, or gene-specific primers.
3. The cDNA for the specific target is amplified by PCR using both a gene-specific probe and a pair of gene-specific primers.
4. The probe has both a fluorescent reporter and a quencher attached to either end so there is no fluorescence signal when the probe is intact.
5. The specific primers and probe anneal to the specific target gene.
6. Due to the 5′-exonuclease activity of the Taq polymerase, the bound fluorescent-labeled probe is degraded while new PCR product is being synthesized. This process releases the fluorescent tag from the quencher and generates a fluorescence signal that is directly proportional to the amount of PCR product in the tube.
7. RT-PCR thermocyclers can detect the abundance of fluorescence and thus determine the relative amount of mRNA present in a given sample.

3.9. Introduction: 21st Century Prognostic and Predictive Factors

Gene expression profiling defines, at the molecular level, the unique gene expression inherent to many kinds of tumors. One of the common features of these studies has been the emergence, through *unsupervised*, hierarchical clustering analysis, of breast cancer subtypes with distinct gene expression patterns for each subtype. The differences in thousands of individual gene expression patterns among these subtypes likely reflect basic differences in the cell biology of the tumors; and therefore, these molecular subtypes may be considered as separable diseases. The molecular differences between the tumor subtypes are often accompanied by differences in clinical features, such as statistically robust differences in relapse-free and OS *(44,57)*. This certainly is not a new idea. As early as 40 years ago, Fox noted in JAMA that the survival curves of women with breast cancer suggests that two or more populations exist, with about 40% suffering fatal outcome unaffected by treatment. The remaining 60% exhibit a relative mortality only modestly different from that of women of similar ages without evidence of disease. He speculated that increasing detection of an entity that is histologically defined as malignant but biologically relatively benign could account for the observed increase in incidence *(23)*.

When an alternative approach, analysis *supervised* by outcome data, is used many groups identify smaller numbers of individual genes whose expression is associated with prognosis and treatment response *(42,61)*. These genes define potential prognostic and predictive molecular markers without respect to the biological diversity represented by the subtypes.

4. A NEW BREAST TAXONOMY: MOLECULAR SUBTYPES USING UNSUPERVISED CLASSIFICATION

4.1. Unsupervised Molecular Taxonomy of Breast: Luminal A/B, HER2, and Basal Subtypes of Breast Carcinoma

Perou and Sorlie first reported a molecular taxonomy of breast cancer based on variation in global gene expression patterns measured by cDNA microarrays, using *unsupervised* statistical correlations in expression patterns. This demonstrated that tumors could be grouped into molecular types distinguished by unique gene expression patterns. The types include luminal A and B (ER positive), HER2 (Erb-B2 positive), and basal type (ER/PR/HER2 negative or "triple

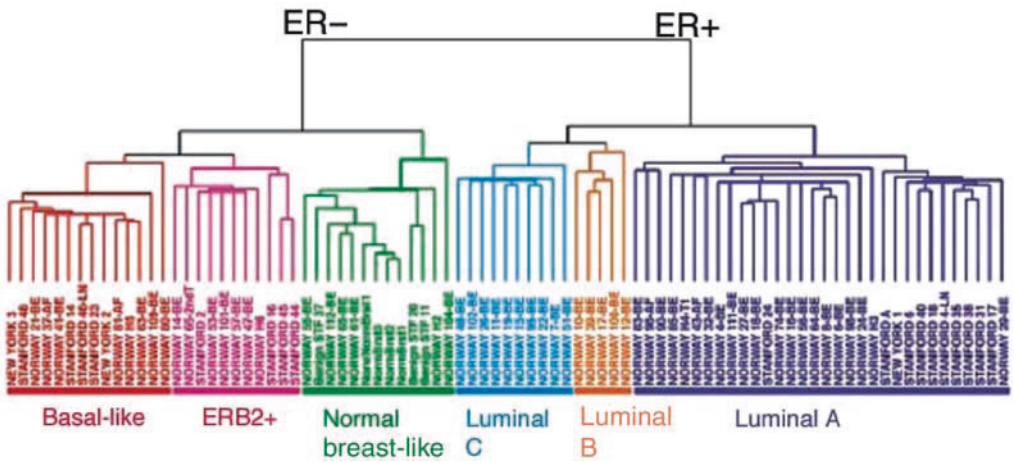

Fig. 11. Gene expression patterns of 85 experimental samples representing 78 carcinomas, three benign tumors, and four normal tissues, analyzed by hierarchical clustering. The closer samples are together, the more similar are their expression profiles. The tumor specimens were divided into six subtypes based on differences in gene expression. Note differences in ER– and ER + . The cluster dendrogram shows the six subtypes of tumors from *left* to *right* (colored as): basal-like, *red*; ERBB21, *pink*; normal breast-like, *green*; luminal subtype C, *light blue*; luminal subtype B, *yellow*; luminal subtype A, *dark blue(57)*. (*see* Color Plate 32)

negative"). These types differ in disease outcome and therapeutic response. In further studies using a directed or *supervised* approach, they identified and attempted to reconcile the definition of those subtypes and the accompanying differences in disease outcome (Fig. 11 and Color Plate 32). Perhaps most exciting about these elegant studies was the identification of two types of ER + breast carcinoma with differing outcomes. The luminal A type breast cancers patients were noted to have significantly better clinical outcomes with regard to recurrence and OS than those ER + tumors classified as luminal B.

4.2. Confirmation of Taxonomy: Evaluation of Breast Cancer Data from van't Veer et al.

Van't Veer et al. used gene expression data from 24,480 genes in a set of 117 tumors from young breast cancer patients. Again a supervised method of hierarchical clustering was used, exactly as described for the Perou/Sorlie data, to display the expression patterns of 461 *intrinsic* genes in the 97 tumor samples that were obtained from patients diagnosed with sporadic cancer (Fig. 12 and Color Plate 33). Individual dendrogram branches are colored according to the strongest correlation of the corresponding tumor with the subtype centroid as defined for the Perou/Sorlie samples. As in the Perou/Sorlie data, the best discrimination was between tumors that expressed genes in the luminal A cluster at high levels (many related to ER and ER-associated genes) and the tumors that were negative for these genes and exhibited expression profiles characteristic of either the basal or the HER2 types. All samples that showed the strongest correlation with the basal subtype (red branches) are all contained within the left branch of the dendrogram in a tight cluster. The luminal A/luminal B distinction, though much less clear than the basal versus luminal distinction, is also seen, with many of the luminal B tumors clustering near each other on the right branch of the dendrogram.

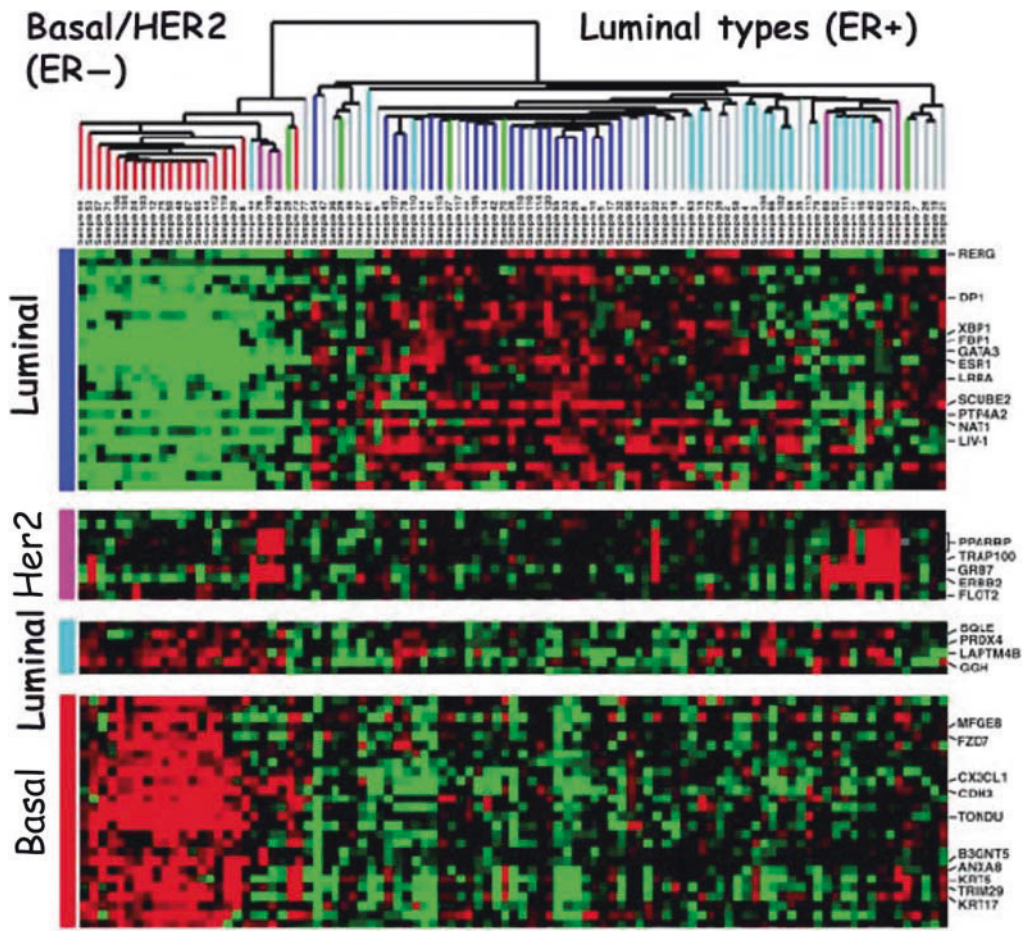

Fig. 12. Individual dendrogram branches are colored according to the strongest correlation of the corresponding tumor with the subtype centroid as defined for the Perou/Sorlie samples. The best discrimination was between the tumors of the luminal A cluster at high levels and tumors that exhibited expression profiles characteristic of the basal, HER2 or luminal B subtypes. The strongest correlation was with the basal subtype (*red branches*)—all of which are contained within the *left* branch of the dendrogram in a tight cluster. (*see* Color Plate 33)

4.3. Tumor Subtypes Are Associated with Significant Difference in Clinical Outcome

In the previous work of Sorlie et al., the expression-based tumor subtypes were associated with a significant difference in OS as well as disease-free survival for the patients suffering from locally advanced breast cancer and belonging to the same treatment protocol. To investigate whether these subtypes were also associated with a significant difference in outcome in other patient cohorts, they performed a univariate Kaplan–Meier analysis with time to development of distant metastasis as a variable in the data set comprising the 97 sporadic tumors taken from van't Veer et al. As shown in (Fig. 13 and Color Plate 34), the probability of remaining disease-free was significantly different between the subtypes; patients with luminal A type tumors lived considerably longer before they developed distant disease, whereas the basal and HER2 groups showed much shorter disease-free time intervals. The methodological differences prevent a definitive interpretation, but it is notable that the order of severity of clinical outcome associated with the several subtypes is similar in the two dissimilar cohorts. This implies that these differences are real and are due to difference in tumor biology.

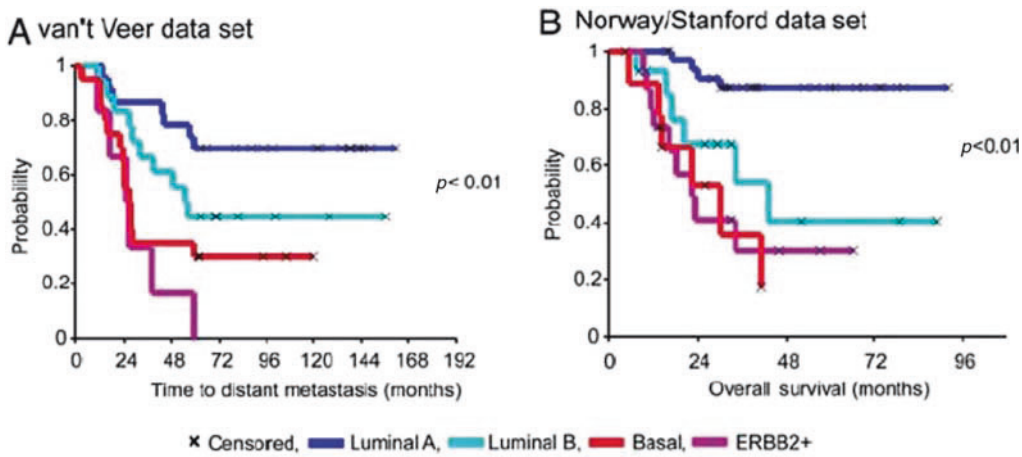

Fig. 13. Kaplan-Meier analysis of disease outcome in two patient cohorts. (**A**) time to development of distant metastasis in the 97 cases from van 't Veer et al. Patients were stratified according to the subtypes as shown in Fig. 14. (**B**) Overall survival for 72 patients with locally advanced breast cancer in the Norway cohort. The normal-like tumor subgroup was omitted in both analyses. (*see* Color Plate 34)

4.4. Breast Tumor Subtypes Represent Reproducible Distinct Biological Entities

Gene expression studies show that there is considerable diversity among breast tumors, both biologically as well as clinically. This is not a new idea, as epidemiological studies previously had inferred the existence of two or more subpopulations of breast cancer *(23)*. A parsimonious interpretation of the reproducibility of several different patterns of gene expression is to regard each as representing a different biological entity. One exciting possible basis for the differences in these patterns between tumor subtypes may be that they originate from different mammary epithelial cell types. The findings support this interpretation, as breast tumor subtypes with patterns of gene expression similar to those of luminal epithelial cells (the cells that line the duct and give rise to the majority of breast cancers) and patterns of at least one other subtype (basal) seem to resemble the pattern found in basal epithelial cells of the normal mammary gland (characterized by expression of cytokeratins 18 and 19 for luminal cells and cytokeratins 5/6 and 17 for basal cells).

4.5. Conclusion: New Breast Taxonomy

Luminal and basal tumor subtypes appear to be distinct biological entities, as the expression patterns have been show to be detectable in other genome-scale studies of breast cancer. Sorlie et al. have found strong evidence for the universality of a distinction between basal-like and luminal-like subtypes in three independent data sets comprising different patient populations whose gene expression profiles had been determined by using different microarray technology platforms. They also found considerable evidence, in one of the studies, for the distinction between the luminal A and B subtypes. The fact that these distinctions for the basal and luminal subtypes are reproducible (less so for the luminal B subtype vs. luminal A) means that the substantial differences in the characteristics of the patients (e.g., age and tumor stage) are less important determinants of tumor expression phenotypes than intrinsic biology.

5. GENOMIC CLASSIFIERS: PROGNOSTIC AND PREDICTIVE SIGNATURES

5.1. Introduction

The first published breast cancer classifier was that of van't Veer et al. from the Netherlands. This was followed by an independent validation study and a subsequent validation study. This work resulted in a commercially available clinical product, Agendia's MammoPrint assay. Similarly, the Genomic Health Onco*type* DX assay was developed, refined, and independently validated. It too resulted in a commercially available clinical product. There are other classifiers in various stages of development and validation. These include a two gene ratio from Sgroi et al., a 76 gene classifier from Veridex *(22)* as well as a classifier being developed by Celera. For the purposes of this discussion, we will limit it to the first two classifiers, which have been independently validated in more than one study, and which are both being used in two large prospective trials, in Europe (the MammoPrint assay in MINDACT: microarray in node negative disease may avoid chemotherapy) and the USA (the Onco*type* DX assay in TAILORx: trial assigning individualized options for treatment). The discussion will provide the basis for evaluating these and other emerging classifiers.

5.2. Classifier Development (54)

There is a large body of literature on prognostic factors for cancer patients unfortunately only a few are used in clinical practice. They are unlikely to be used unless they are therapeutically relevant and most publications do not establish such therapeutic relevance. Most prognostic factor studies are conducted by use of a convenient sample of patients for whom tissue is available, and the cohort is often far too heterogeneous with regard to age, ER status, stage, and treatment to support therapeutically relevant conclusions *(54)*.

As there are many new classifiers in development, it is useful to divide genomic classifier studies into developmental studies and validation studies. Developmental studies define the genes and the algorithms used in multigene classifiers and are analogous to phase I–II clinical trials while validation studies that use prespecified genes, algorithms, and analysis plans are analogous to phase III clinical trials.

In development studies, one significant concern is that the number of candidate genes studied by DNA expression arrays for use in the classifier is typically much larger than the number of cases available for analysis. In these studies it is always possible to find genes or sets of genes that perfectly classify the data on which they were developed. This apparently *perfect* classification can be achieved even if there is no relationship between expression of any of the genes and outcome. Therefore, even in developmental studies, both control for false discovery of genes and validation of the selected genes and classifier is necessary. "Internal validation" may be accomplished by (1) splitting the data into two portions, one used for training the model and the other used for testing the model; or (2) cross-validation that is based on repeated model development and testing on random data partitions. However, neither of these statistical methods provides true "independent" validation. Thus, these methods for *internal* validation do not constitute *external* validation of the classifier in a setting simulating broad clinical application.

In an editorial to JNCI, Dr. Simon, Chief, Biometric Research Branch, NIH recommends the following roadmap for developing a genomic classifier:

- Developmental and validation studies should be based on cohorts of patients that are sufficiently homogeneous for therapeutically relevant classifiers to be developed. . .this is best achieved by studying patients who were included in a single, large, randomized clinical trial.

- Developmental studies should be sufficiently large so that they can incorporate either cross-validation or split sample validation and demonstrate that the internally validated prediction error is statistically significantly less than would be expected by chance.
- Independent validation studies are essential before results are accepted into medical practice.
- Independent validation studies should apply the classifier completely specified, including cutoffs, by the developmental study and measure prediction accuracy.
- The size of the validation study should be sufficient so that meaningful confidence intervals on predictive accuracy and positive and negative predictive values can be reported.
- The size of the validation study should be sufficient so that the extent to which the classifier adds predictive accuracy to established prognostic factors can be meaningfully evaluated.

6. THE MAMMOPRINT BREAST CANCER ASSAY

6.1. Breast Cancer Classifier: van't Veer et al. (59)

The Netherlands group identified a 70 gene classifier developed using expression microarrays with ~25,000 human genes from fresh tissues obtained from primary breast tumors of 117 young female patients. This group used supervised classification to identify a 70 gene expression signature strongly predictive of a short interval to distant metastases (poor prognosis signature) in 78 of the lymph node-negative patients.

To do this they selected 98 primary breast cancers and tested ~25,000 genes which clustered the tumors on the basis of gene expression. All sporadic patients were node negative and under 55 years of age. RNA was isolated from frozen tissues and was used to derive complementary RNA (cRNA). A reference pool was made from a pool of all sporadic carcinomas. Two hybridizations were made for each tumor using a fluorescent dye reversal technique on microarrays synthesized by inkjet technology on arrays containing approximately 25,000 human genes. Approximately 5,000 genes were significantly regulated across the group of samples (p value of less than 0.01 in more than five tumors). Using unsupervised, hierarchical clustering, they clustered the 98 tumors on the basis of their similarities. They found two distinct groups, one with a poor prognosis with distant metastases and another good prognosis group without progressive disease. To gain insight into the genes of the dominant expression signatures they found that the ER– cases clustered together and there was a second group associated lymphocytic infiltrate, including several genes expressed primarily by B and T lymphocytes.

They then used 78 sporadic lymph node negative tumors to search for a prognostic signature in their gene expression profiles. Forty four remained disease free for an interval of at least 5 years (good prognosis group) and 34 patients that developed metastases within 5 years (poor prognosis group) (Fig. 14 and Color Plate 35).

The 70-gene classifier was identified by using a three-step supervised classification method. The accuracy was improved until the optimal number of genes, 70, were identified. This classifier predicted correctly the actual outcome of disease for 65 out of the 78 patients (83%), with, respectively, 5 poor prognosis and 8 good prognosis patients assigned to the opposite category. An optimized threshold was established by optimizing the algorithm so that no more than 10% of poor prognosis patients were misclassified. This optimized sensitivity threshold resulted in 15 misclassifications: 3 poor prognosis were classified as good and 12 good prognosis tumors were classified as poor.

The upregulated genes associated with a poor prognosis included those involved in cell cycle, invasion and metastasis, angiogenesis, and signal transduction (e.g., cyclin E2, metalloproteinases MMP9 and MMP1, and the VEGF receptor FLT1). The prognosis classifier was validated in an additional, independent set of 19 young, node-negative

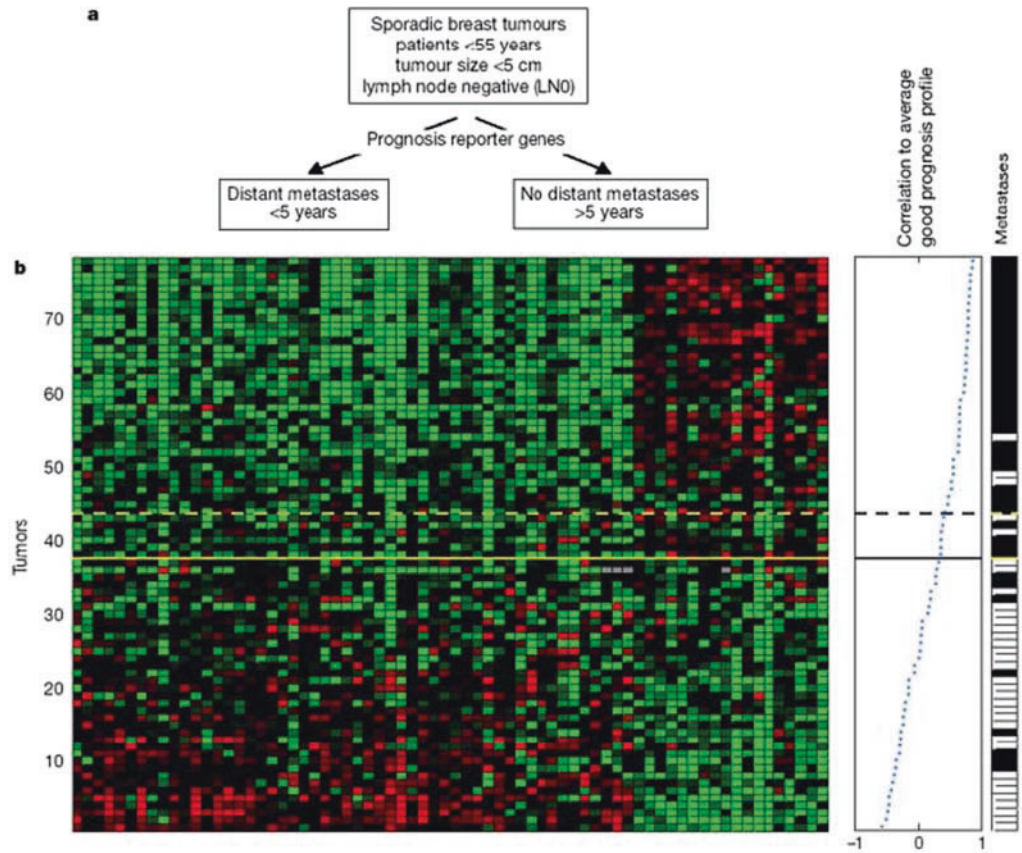

Fig. 14. Supervised classification on prognosis signatures. (**A**) Prognostic reporter genes identify optimally two types of disease outcome from 78 sporadic breast tumors into a poor prognosis and good prognosis group. Each row represents a tumor and each column a gene. Genes are ordered according to their correlation coefficient with the two prognostic groups. The *solid line* represents the prognostic classifier with optimal accuracy; *dashed line* with optimized sensitivity. Above the *dashed line* are patients with a good prognosis and below those with a poor prognosis. The metastasis status for each patient is shown in the *right panel*: white indicates patients with metastases within 5 years and black indicates those disease free for at least 5 years. (*see* Color Plate 35)

patients. This group consisted of seven patients who remained disease free for at least 5 years and 12 patients who developed distant metastases within 5 years. The disease outcome was predicted by the 70-gene classifier and resulted in 2 out of 19 incorrect classifications using the optimized threshold.

The prediction of the classifier presented would indicate that women under 55 years of age who are diagnosed with lymph node-negative breast cancer that have a poor prognosis signature have a 28-fold odds ratio (OR) (95% confidence interval, 95% CI 7–107, $p = 1.0 \times 10^{-8}$). This classifier provided additional prognostic information in addition to the traditional clinical and histopathological prognostic factors: high grade (OR = 6.4, 95% CI 2.1–19, $p = 0.0008$), tumor size greater than 2 cm (OR = 4.4, 95% CI 1.7–11, $p = 0.0028$), angioinvasion (OR = 4.2, 95% CI 1.5–12, $p = 0.01$), age less than or equal to 40 years (OR = 3.7, 95% CI 0.9–6.6, $p = 0.13$), and ER– (OR = 2.4, 95% CI 0.9–6.6, 95% CI 0.9–6.6, $p = 0.13$). Multivariate analysis that included all classical prognostic factors indicated that it was an independent factor in predicting outcome of disease (logistic regression OR = 18, 3.3–94, $p = 1.4 \times 10^{-4}$).

6.2. Validation of the 70-Gene Classifier (60)

Using fresh frozen tissue and microarray analysis to evaluate the previously established 70-gene prognosis profile, the Netherlands group classified a series of 295 consecutive patients with primary breast carcinomas as having a gene expression signature associated with either a poor prognosis or a good prognosis. All patients had stage I or II breast cancer and were younger than 53 years old; 151 had lymph node-negative disease, 144 had lymph node-positive disease; 155 had tumors less than or equal to 2.0 cm, 140 had tumors greater than 2.0 cm; 99 were ER– and 226 were ER + ; 185 received adjuvant chemotherapy, 110 did not; and 40 received hormonal therapy and 255 did not. They evaluated the predictive power of the prognosis profile using univariate and multivariate statistical analyses.

Among the 295 patients, 180 were found to have a poor prognosis signature and 115 had a good-prognosis signature, and the mean (±SE) overall 10-year survival rates were 54.6 ± 4.4 and $94.5 \pm 2.6\%$, respectively. At 10 years, the probability of remaining free of distant metastases was $50.6 \pm 4.5\%$ in the group with a poor-prognosis signature and $85.2 \pm 4.3\%$ in the group with a good-prognosis signature (Fig. 15 and Color Plate 36). The prognosis profile was significantly associated with tumor histologic grade ($p < 0.001$), the ER status ($p < 0.001$), and age ($p > 0.001$) but not tumor size, extent of vascular invasion, the number of positive lymph nodes, or treatment.

The estimated hazard ratio (HR) for distant metastases in the group with a poor-prognosis signature, as compared with the group with the good-prognosis signature, was 5.1 (95% CI 2.9 – 9.0; $p < 0.001$). This ratio remained significant when the groups were analyzed according to lymph node status (Fig. 16 and Color Plate 37). The prognosis profile was a strong independent factor in predicting disease outcome (HR 5.5 among those with poor-prognosis signature compared to those with a good signature). The profile was also strongly associated with the outcome in the group of 144 patients with lymph node-positive disease, HR for distant metastases = 4.5 (95% CI 2.0–10.2; $p < 0.001$) (Fig. 16).

Between the two studies, the Nature study and the NEJM study, in lymph node negative patients, the ORs for the development of distant metastases within 5 years were similar, 15.3 and

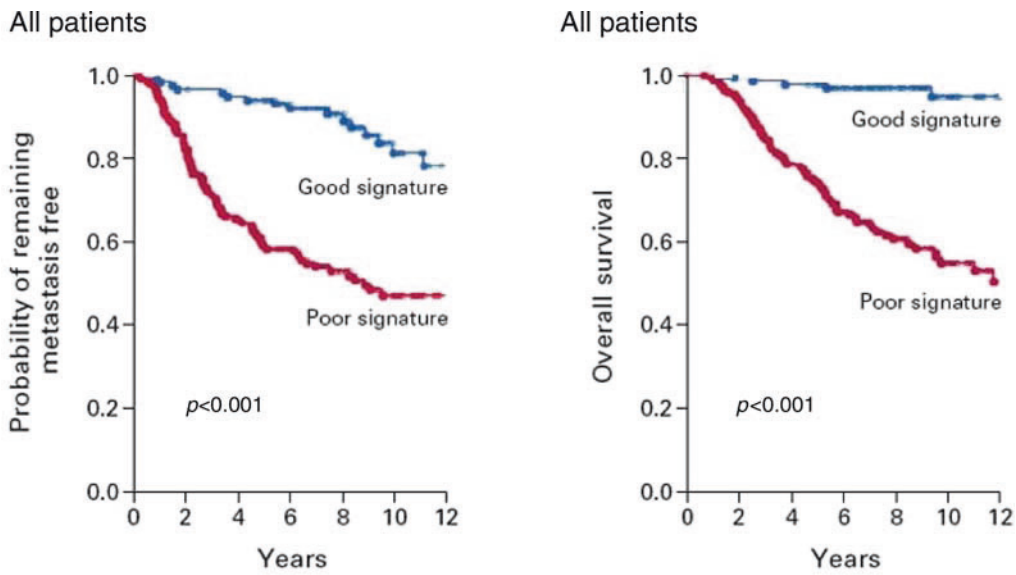

Fig. 15. Kaplan–Meier analysis of the probability that patient would remain free of distant metastases and the probability of overall survival among all patients. (*see* Color Plate 36)

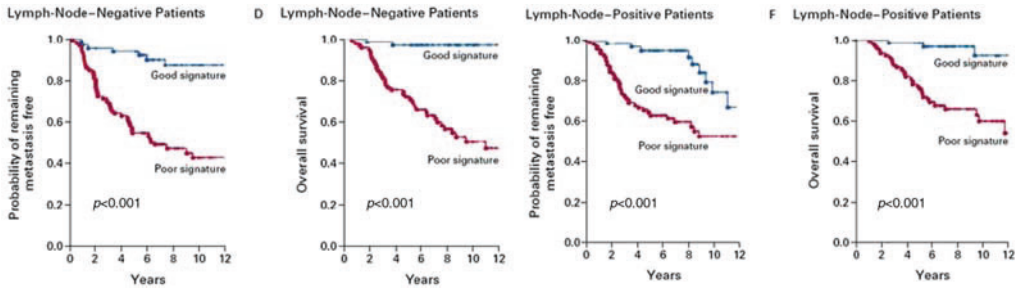

Fig. 16. Kaplan–Meier analysis of the probability that lymph node-negative patient (*left two panels*) and lymph node-positive patients (*right two panels*) would remain free of distant metastases and the probability of OS, respectively. (*see* Color Plate 37)

15.0. The prognosis signature was also highly predictive of the risk of distant metastases among the subgroup of patients with lymph node positive disease, which were not present in the original Nature study. The authors conclude that the gene expression profile is a more powerful predictor of the outcome of disease in young patients with breast cancer than standard systems based on clinical and histologic criteria.

6.3. Criticisms

The study has been criticized by many statisticians because of the inclusion of patients in the development set in the validation cohort. In an independent multicenter validation study, presented at the 2004 San Antonio Breast Cancer Symposium, the 70-gene prognostic index performed less well than in previous studies, although it was still prognostic (note that in a Breast Cancer International Group [BIG] newsletter [Vol. 7(3), 2005]), reanalysis of the data has been claimed to show a much more robust prognostic power). BIG is launching a large clinical trial called MINDACT ($n = 6{,}000$ patients) based on upfront stratification using this 70-gene assay, now available as a commercial reference laboratory test, called MammaPrint, in Europe (by Agendia). It is currently available in the USA and has received FDA 510(k) approval *(40)*.

7. ONCO TYPE DX^{TM}

7.1. Introduction: Onco type DX^{TM}

The Onco*type* DX^{TM} breast cancer assay is a QRT-PCR assay for fixed paraffin-embedded samples. The assay is clinically validated to assess the risk of 10-year distant recurrence, the likelihood of patient survival within 10 years of diagnosis, and the magnitude of chemotherapy benefit. This test is currently validated for women with early-stage, node negative, ER+ (N–, ER+) breast cancer who will be treated with hormonal therapy. The assay quantifies the expression of 21 genes using TaqMan^{TM} (quantitative PCR). The assay was clinically validated in a large, multicenter clinical trial with prospectively defined endpoints, conducted by the National Surgical Adjuvant Breast and Bowel Project (NSABP), and the assay results were confirmed in a community-based patient population study with Northern California Kaiser Permanente.

7.2. Assay Design

There were four phases in the design Onco*type* DX^{TM}: (1) technical feasibility studies, (2) development studies, (3) analytical validation, and (4) clinical validation studies.

7.3. Technical Feasibility Studies

These studies demonstrated the ability to reproducibly extract mRNA from fixed paraffin-embedded tissues from archival tissues up to 30 years in age, developed normalization strategies to account for increasing RNA degradation over time, resolved issues of tumor heterogeneity, and determined objective cut points for tumor manual microdissection.

7.4. Development Studies

In the development process, 250 candidate genes were selected from the published literature, genomic databases, and experimental microarray data for breast cancer. The genes were tested in three independent studies including cases from NSABP B-20, in total, involving 447 patients. Multivariate analyses indicated that panels of multiple genes had greater predictive power than any single gene. The data from all three studies were used to select a 21-gene panel (16 cancer-related genes and 5 reference genes) that strongly and consistently correlated with likelihood of distant recurrence. The recurrence score (RS) is used to predict patient prognosis, it ranges from 0 to 100 and it is divided into three risk groups: (1) a low-risk score correlating with a low risk of distant recurrence, RS 0–18; (2) an intermediate risk score correlating with an intermediate risk of distant recurrence, $18 < RS < 31$; and (3) a high-risk score correlating with a high risk of distant recurrence, $RS \geq 31$.

7.5. Recurrence Score Calculation

The RS calculation is as follows: RS = + 0.47 × HER2 group score – 0.34 × ER group score + 1.04 × proliferation group score + 0.10 × invasion group score + 0.05 × CD68 – 0.08 × GSTM1 – 0.07 × BAG1. Although some of the coefficients are greater than others, each of the individual genes can greatly influence the individual RS.

7.6. Clinical Validation Studies

7.6.1. NSABP B-14: Introduction

NSABP B-14 was a landmark clinical trial that established the value of tamoxifen in hormone receptor-positive breast cancer patients. This validation study was carefully designed to meet rigorous statistical standards and included a prospectively defined gene list and RS calculation, prospectively defined RS cutoffs for the risk groups, prospectively defined endpoints and a prospectively defined analysis plan. The NSABP controlled the clinical data and the data analysis. In the validation study, the assay was found to provide a better and/or more reproducible indication of prognosis for ER+ tumors in node-negative patients than age, tumor size, or histologic grade. The quantitative data allow an individualized risk estimate to be derived (on a scale of 0–100), which is a significant improvement over classical prognostic indicators.

7.7. Study Design

The B-14 study involved 668 evaluable patients: those B-14 participants for whom both tissue and clinical follow-up data were available. All these patients were stage I or II, node negative, ER+, and received tamoxifen treatment. The 668 patients were similar in terms of age distribution and the distribution of tumor size to the entire group of 2,617 tamoxifen-treated patients. Patients from the tamoxifen treatment arm were chosen, instead of those from the placebo arm, because hormonal therapy such as tamoxifen is the standard of care for women with ER+ breast cancer.

7.8. Methodology

For each patient, three 10-μm formalin-fixed, paraffin-embedded tissue specimens from the NSABP B-14 baseline tumor samples were sent to Genomic Health, Inc. (GHI) in a blinded fashion. For each specimen, the 21-gene RT-PCR assay was performed, and the Recurrence Score™ calculated without the knowledge of the patients' clinical outcomes.

7.9. Analysis

The prespecified endpoints were as follows: the primary endpoint was distant recurrence-free survival (DRFS), while the secondary endpoints included relapse-free survival (RFS) and OS. The clinical outcomes were compared with the measurement for each individual of their RS. The comparison was conducted in a blinded fashion.

7.10. Results

Of note is that in this "retroprospective study" paraffin blocks from NSABP trial B-14, with median follow-up of more than 14 years, were used. The technical success rate of the Oncotype DX™ assay was 99% (of the 675 patients who were eligible (pathology and clinical), insufficient RNA or RT-PCR outside of specifications occurred in seven patients (1%). Thus 668 or (99%) of the patients were evaluable in the final analysis). The study met its prospectively defined endpoints: RSs were independent and highly significant predictors of recurrence-free survival and RS provided accuracy and precision in predicting likelihood of distant recurrence ($p < 0.001$). Assignment to the risk groups accurately predicted distant recurrence at 10 years (Fig. 17 and Color Plate 38).

The RS performance was shown to exceed standard measures, such as age, tumor size, and tumor grade either in predictive power or in reproducibility (Table 2). There is a near-linear relationship between the numerical RS and the patient's actual risk of distant recurrence.

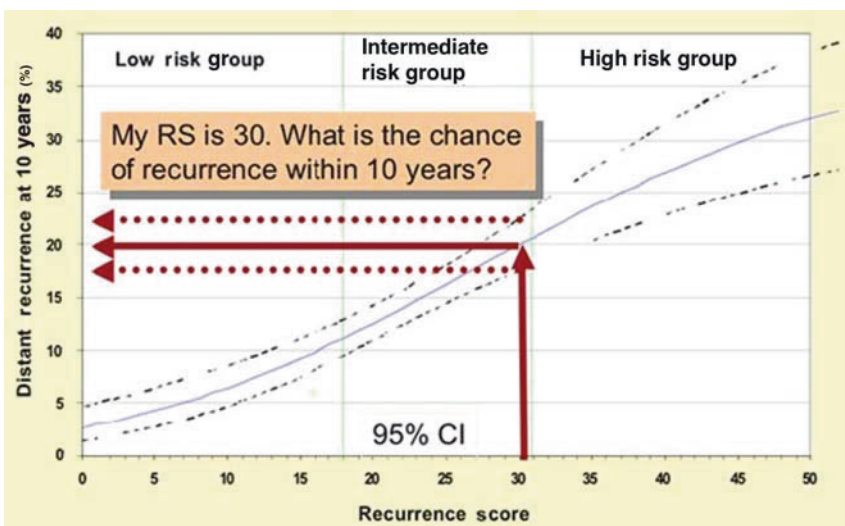

Fig. 17. The recurrence score (RS) is used to predict patient prognosis, it ranges from 0 to 100 and it is divided into three risk groups: (1) a low-risk score correlating with a low risk of distant recurrence, RS 0–18; (2) an intermediate risk score correlating with an intermediate risk of distant recurrence, $18 < RS < 31$; and (3) a high-risk score correlating with a high risk of distant recurrence, $RS \geq 31$. (*see* Color Plate 38)

Table 2
Multivariate Cox proportional hazards analysis examining all the standard measures alone and then in combination with the Oncotype DX™ Recurrence Score

	Analysis without RS		*Analysis with RS*	
Variable	*p value*	*Hazard ratio*	*p value*	*Hazard ratio*
Section 34.05 Age at surgery	0.1	0.7	0.22	0.76
Clinical tumor size	0.13	1.35	0.38	1.19
Tumor Grade				
Moderate	0.04	1.87	0.15	1.55
Poor	<0.001	5.14	<0.001	3.34
HER2 amplification	0.89	1.04	0.06	0.51
ER protein				
50–99 fmol/mg	0.23	0.71	0.32	0.75
100–199 fmol/mg	0.38	0.78	0.72	0.9
>200 fmol/mg	0.9	0.97	0.94	1.02
Recurrence score			<0.001	2.81

Shown here is a multivariate Cox proportional hazards analysis examining all the standard measures alone (including age, tumor size, tumor grade, HER2, and ER expression), and then the standard measures in combination with the Oncotype DX™ Recurrence Score. Only the RS and poor tumor grade are independently associated with recurrence.

7.11. Additional Validation Study: Kaiser Permanente Study

An additional validation study of in 220 cases and 570 controls, conducted by the Northern California Kaiser Permanente in its community-based patient set, confirmed the results from the NSABP B-14 study, that the RS was statistically significantly associated with breast cancer survival in tamoxifen-treated patients with node-negative, ER + breast cancer ($p = 0.0002$). The risk of breast cancer death for patients in the low-risk group as determined by the RS (RS < 18) was 2.8% at 10 years. Moreover, over 50% of the patients were it the low-risk group. It is notable that this genomic assay can identify such a large cohort of patients who retain such a low risk of breast cancer death.

7.12. Prediction of Chemothrerapy Benefit: NSABP B-20

NSABP B-20 also assessed the assay's ability to predict the magnitude of benefit from chemotherapy.This study examined the performance of the Onco*type*DX™ assay in tumor blocks from 651 patients enrolled from 1988 to 1993 in the tamoxifen alone and tamoxifen plus either cyclophosphamide, methotrexate and fluorouracil (5FU) (CMF) or methotrexate and fluorouracil (5FU) (MF) chemotherapy treatment randomization arms of the NSABP Study B-20.

7.13. Results

Patient-specific RSs derived from the multigene assay were obtained for a total of 651 eligible patients from the tamoxifen alone arm ($n = 227$) and the tamoxifen plus chemotherapy treatment arm ($n = 424$) of B-20. Cox proportional hazards models for the global test of interaction between treatment effect (either tamoxifen alone or tamoxifen plus chemotherapy) and gene expression reveal that the Recurrence Score is a significant predictor of chemotherapy benefit ($p = 0.038$).

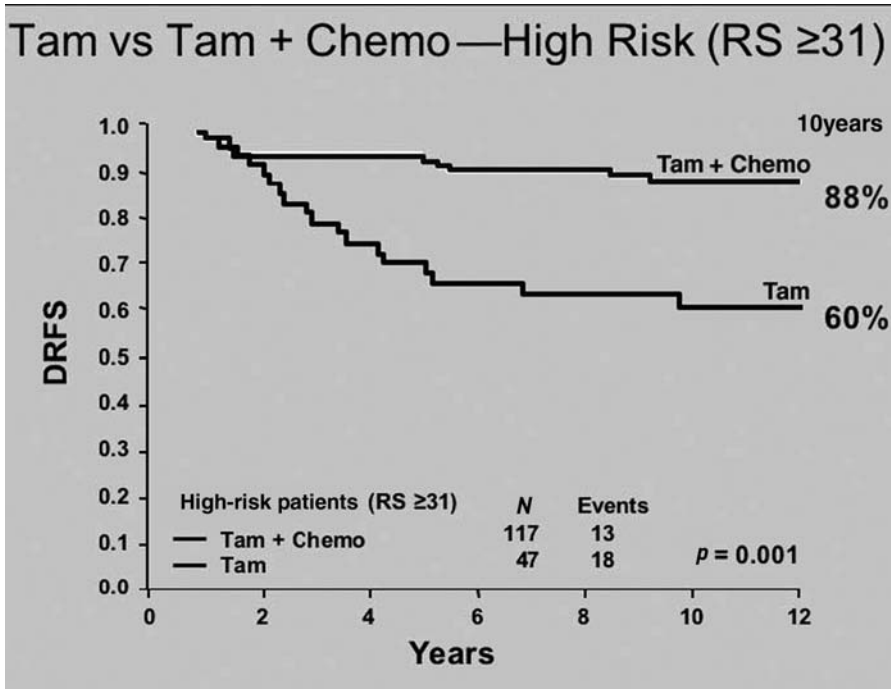

Fig. 18. Not all patients benefit equally from chemotherapy. Patients with low RS tumors derived minimal benefit from chemotherapy while patients with high-risk tumors had a 28% absolute benefit from chemotherapy.

As the RS increases, the likelihood of chemotherapy benefit increases. Not all patients benefit equally from chemotherapy. Patients with low RS tumors (RS < 18) derived minimal, if any, benefit from chemotherapy (an estimated increase in DRFS at 10 years of $-1.1 \pm 2.2\%$, mean $\pm$ SE). Patients with high-risk tumors (RS ≥ 31) had a large absolute benefit of chemotherapy (an absolute increase in DRFS at 10 years of $27.6 \pm 8.0\%$, mean $\pm$ SE) (Fig. 18).

RS predicted the absolute risk of breast cancer death at 10 years (low RS = 2.8% [95% CI 1.7%, 3.9%]; intermediate RS = 10.7% [95% CI 6.3%, 14.9%]; high RS = 15.5% [95% CI 7.6%, 22.8%]). These risk estimates were similar to those in the NSABP B-14 Clinical Validation Study.

7.14. Reclassification Versus Guidelines

Analysis of the 668 patients from the B-14 study compared the patient Recurrence Scores with patient risk of distant recurrence as determined by clinical guidelines. Overall, 47% of patients were reclassified by Onco*type* DX^{TM} when compared to NCCN guidelines.

8. CONCLUSION

Significant progress has been made in the use of DNA microarray and QRT-PCR analysis in developing robust genomic classifiers that will compliment the current prognostic metrics that as assessed today. The hope is that these and yet to be defined platforms and gene combinations, either as ratios, centroids, or equations will define personalized medicine by providing prognostic and predictive tools to assist in 21st century clinical decision making.

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41 Molecular Signatures in Melanoma Progression

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CONTENTS

ABSTRACT

Since melanoma is a heterogenous disease, prognosis based on molecular markers may provide important insight into the biologic behavior of melanomas and may have even greater significance for predicting survival, recurrence risk, lymph node involvement, and distant metastasis when compared with current prognostic measures. This chapter describes the molecular basis and evolution of melanoma from radial growth phase to vertical growth phase and examines predictors of lymph node metastasis. Additionally, the molecular signatures from nevus to primary melanoma to metastatic melanoma will be described.

Key Words: gene expression; molecular markers; thin melanomas; sentinel lymph node metastasis; radial growth phase; vertical growth phase

1. INTRODUCTION

Patients with thin melanomas (<1.0 mm) generally have an excellent prognosis, with a relapse rate of less than 10% at 5 years and approaching a relapse rate of less than 1% in melanoma in situ lesions *(2)*. Patients with stage IV disease have about a 10% 5-year survival. However, patients with thin melanomas can succumb to metastatic disease, a phenomenon that is well documented clinically and incompletely characterized at the molecular level.

Prognostic factors are derived from clinical and histological features and the emerging area of molecular studies. The revised AJCC guidelines for melanoma staging incorporated several

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changes based on an analysis of clinical and histological prognostic factors in 17,600 patients with melanoma. Multivariate analysis of prognostic features in primary localized melanoma (stage I and II) included 13,600 patients and 4,750 patients who underwent either elective lymph node dissection (ELND) or sentinel lymph node (SLN) biopsy *(2)*.

For the primary melanoma, tumor thickness (T) was most predictive of survival followed by the presence or absence of ulceration. Clark's level does have prognostic importance and management implications in thin melanomas as previously observed *(31)*. Ulceration upstaged the tumor to the next level without ulceration, a phenomenon described over 25 years ago *(3)*. In patients with nodal disease, N (nodal) status now includes the number of nodes (rather than gross dimension) and the subcategory of nodal disease as microscopic or macroscopic. Patients with in-transit/satellite metastasis without nodal disease are now grouped together into stage III disease. The M (metastatic disease) component classifies disease based on anatomic site (visceral vs. nonvisceral) and inclusion of serum lactic dehydrogenase. Cox regression analysis of the 4,750 patients pathologically staged (ELND or SLN biopsy) showed that nodal status (number of metastatic nodes, micro- vs. macroscopic) was the most significant independent predictor of survival followed by tumor thickness and ulceration.

Thickness and presence (or absence) of ulceration are the only histologic criteria included in the 2002 AJCC guidelines. Other histologic prognostic factors that have been shown to have significant predictive value include vascular invasion, tumor vascularity, regression, mitotic index, and microsatellitosis. A multivariate analysis of prognostic factors showed that vascular involvement was the second most important factor (after tumor thickness) in the primary tumor, predicting survival *(27)*. The presence of vascular involvement was associated with an increased risk of melanoma relapse and death and a significantly reduced relapse-free survival (RFS) and overall survival.

Clearly, the presence or absence of histologic criteria currently *not* included in the AJCC staging guidelines, have validity. However, since melanoma is a heterogenous disease, prognosis based on molecular markers may provide important insight into the biologic behavior of melanomas and thus may have even greater significance for predicting survival and risk of recurrence.

2. BIOLOGY OF TUMOR METASTASIS

The majority of melanomas appear to develop de novo on naïve skin and not from precursor nevi *(19,39,10,48,46,41)*. The lifetime risk of a melanocytic nevus transforming into melanoma for a 20-year-old person is estimated to be 1:3,000 in men and 1:10,000 in women *(49)* and in another study estimated to be 1:2,000 *(4)*. The most common subtype of melanoma, termed superficial spreading melanoma (SSM), begins as an intraepidermal proliferation of melanocytes, forming an in situ neoplasia. This initial intraepidermal melanocytic proliferation as well as minimally invasive disease is referred to as the radial growth phase (RGP). Once the melanoma cells form a dominant clone of tumor cells growing into the dermis, the melanoma is now described as having a vertical growth phase (VGP). In the classical model of melanoma progression, formation of the VGP is presumed to be the first step at which the tumor gains metastatic capacity and thus a prerequisite for subsequent metastatic disease. This sequence of events, the Clark model of melanoma progression *(10)*, predicts a stepwise evolution through these phases (Fig. 1). Recent molecular studies have defined more clearly the *genetic signatures* for each phase of transition from nevus to primary melanoma to metastatic melanoma. Gene expression profiles have now characterized the transition from RGP to VGP *(24)*.

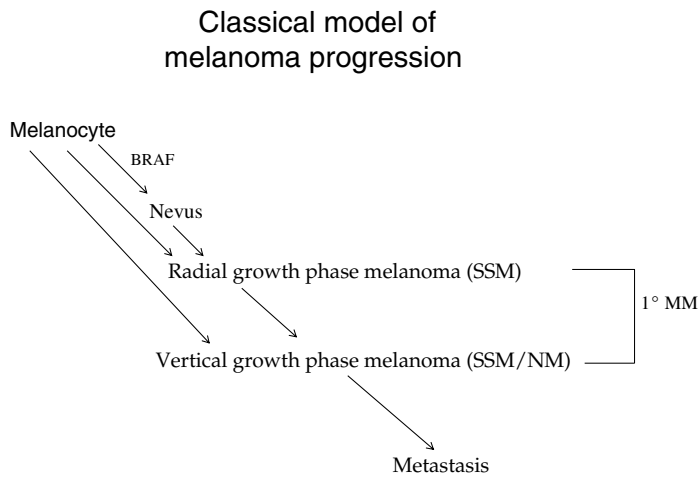

Fig. 1. Model of melanoma progression.

The endpoint of prognosticating is to improve our risk assessment of melanoma patients with the goal of identifying those at increased risk of lymph node involvement and systemic spread. This can be accomplished by the melding of the three classes of prognostic information: clinical, histological and molecular. Ultimately, the molecular characterization of melanomas should also provide important targets for therapy of melanoma metastasis.

3. MOLECULAR SIGNATURES: FROM NEVUS TO MELANOMA

The use of molecular markers in melanoma for prognostic purposes is an emerging and evolving field. Several recent studies have shown that these novel molecular biomarkers may predict disease course superior to current prognostic indicators and also have important implications in subsets of melanoma such as desmoplastic melanoma (DMM) and thin melanomas (<1 mm in thickness).

3.1. B-RAF Activating Mutation as a Precursor

Mutations in components of the MAPK (mitogen-activated protein kinase) signaling pathway, a cell growth cascade, have been implicated in many human cancers. B-RAF, a member of the RAF family of serine/threonine kinases, is an intermediary in the MAPK pathway and has been implicated in the pathogenesis of melanoma *(8,14,35)*. B-RAF mutations have also been found in the primary tumors of several other human cancers including nonsmall-cell lung cancer *(8)*, papillary thyroid cancer *(28)*, and colon cancer *(15)*. Pollack et al. microdissected melanomas (both primary and metastatic) and benign nevi *(35)*. They observed B-RAF mutations in 68% of metastases, 80% of primary melanomas and 82% of benign nevi, implying that B-RAF mutations may be an early event in melanoma development. This has led investigators to seek other biomarkers with the intended ability to distinguish benign nevi from primary melanoma.

3.2. Defining the Molecular Signatures in Melanoma Progression

In a recent study, gene expression profiling with cDNA microarrays was used to characterize the molecular basis of melanoma progression *(24)*. The main objectives of the study included

1) Molecular analysis of the transition from RGP to VGP and validation of these findings in a set of unrelated primary melanomas, then relating these findings to the pathogenesis of metastatic melanoma.

2) Determination if the gene sets can distinguish between a benign nevus and a primary melanoma.
3) Multiclass analysis comparing nevi, primary and metastatic lesions seeking unique profiles of each category of lesion.

3.2.1. Transition from RGP to VGP and Extending the Findings to Metastatic Melanoma

A large primary SSM with demonstrable RGP and VGP was microdissected with laser capture and the RNA from the melanoma cells from these two phases was then hybridized in duplicate arrays and analyzed by SAM (significance analysis of microarrays) to determine the list of genes best able to distinguish radial and vertical growth phases. One may postulate that the transition from RGP to VGP would result in a *gain* of function or *gain* of gene expression allowing for the invasive phenotype (VGP) to develop. In this study, there were in fact, only *losses* of gene expression (Fig. 2 and Color Plate 39). Genes whose expression was lost in the VGP coded for cell adhesion molecules and extracellular matrix proteins, including cadherin-3 (CDH3), matrix metalloproteinase-10 (MMP-10), integrin $\alpha 2$, and laminin $\gamma 2$. Based on this,

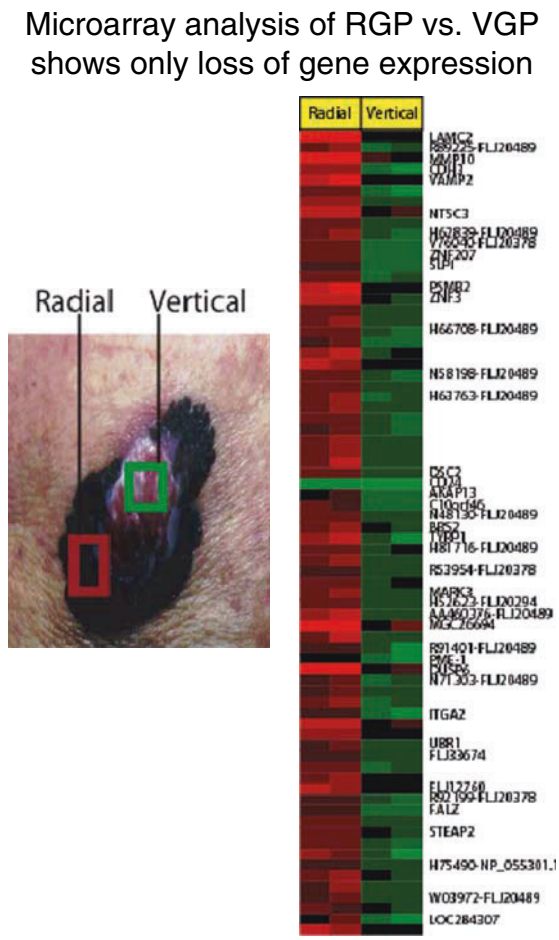

Fig. 2. Microarray analysis of the radial and vertical growth phases within a single, large, primary melanoma. Note that in the vertical growth phase, there is a predominance of *green* signals, indicating losses of gene expression. (*see* Color Plate 39)

CDH3 and MMP-10 immunostaining was performed on 25 unrelated primary melanomas, in which both the RGP and VGP were present. Twenty-two cases had evaluable RGP and VGP for MMP-10. MMP-10 immunostaining was more pronounced in the RGP compared to VGP in 11 cases (Fig. 3a and Color Plate 40); staining was equal in the remaining samples. For CDH3, 19 cases were evaluable for RGP and VGP. Twelve cases showed CDH3 immunostaining to be greater in the RGP than VGP (Fig. 3b) and equal staining in seven cases. The differences in staining of RGP and VGP was statistically significant with a $p < 0.0005$ for CDH3 and $p = 0.001$ for MMP-10. In no case was CDH3 or MMP-10 staining stronger in the VGP compared to the RGP consistent with the gene expression analysis revealing only losses of gene expression during this transition from RGP to VGP. These results confirmed that the transition to VGP is accompanied by loss of gene expression.

3.3. CDH3 and MMP-10

Cadherins are calcium-dependent cell to cell adhesion molecules that have been implicated in the pathogenesis of many different human cancers *(6)*. The cadherin family includes E, N, and P cadherins. The P cadherins (CDH3 gene) are restricted to the basal or lower layers of stratified epithelia of prostate, skin, and breast myoepithelial cells *(32; 44)*. Given the function of cadherins, a loss of adhesion between melanocytes and keratinocytes may play a role in malignant and metastatic behavior, allowing these melanoma cells to circumvent normal structural integrity, gaining the ability to invade. P-cadherins have been shown to portend a poor prognosis in breast cancer and associated with decreased disease-free and overall survival *(33,34,20)*. In an independent study, decreased CDH3 staining correlated with melanoma tumor progression *(43)*.

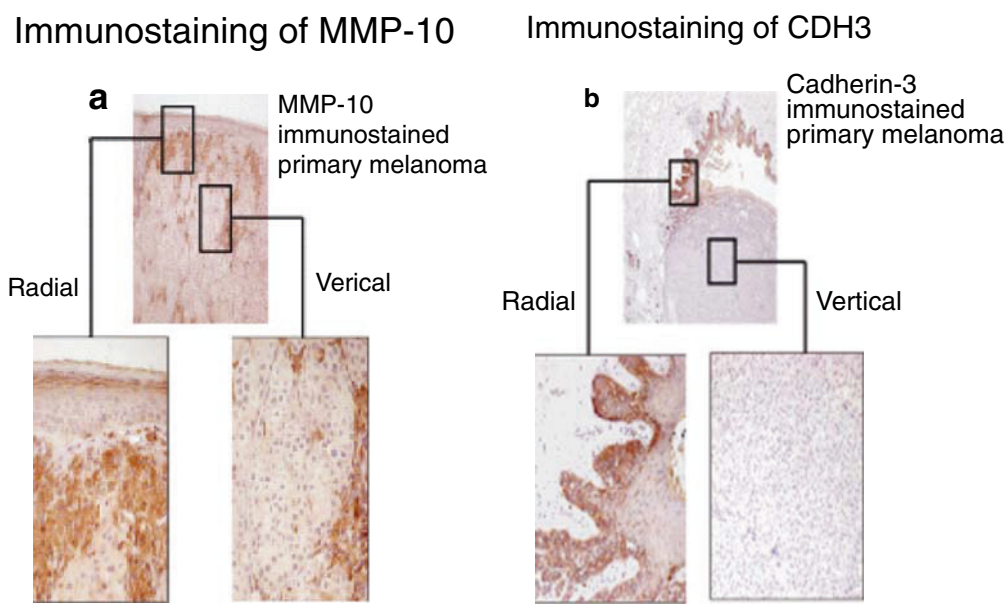

Fig. 3. Representative primary melanoma with evaluable radial and vertical growth phases immunostained for MMP-10 (**a**) and CDH3 (**b**). Prominent staining is noted within the radial growth phase consistent with gene profile analysis showing a loss of these genes in the vertical growth phase. (*see* Color Plate 40)

MMPs are a growing family of at least 20 zinc-dependent endopeptidases (produced as inactive enzymes) that upon activation degrade the extracellular matrix (ECM). The ECM is composed of collagens, elastins, laminins, fibronectins, and proteoglycans. Thus, MMPs are essential for normal cellular activities including tissue remodeling, wound repair, and also during embryogenesis. Active MMPs are regulated by tissue inhibitors of MMPs. Intriguingly, MMPs have also been found to have roles other than simply disintegrating the ECM and have been shown to disrupt cell to cell and cell to matrix adhesion, as well as stimulate cellular proliferation by the release of growth factors contained within the ECM itself *(47)*. In tumorigenesis, proteolysis of the ECM by various MMPs is central to invasion, metastasis, angiogenesis, and endothelial migration *(11,18,26,17)*.

We have had the opportunity to further test these biomarkers in a patient with melanoma in situ with extensive regression and subsequent metastasis. These lesions contain RGP only and are devoid of VGP, either because it was never present or because it was destroyed by the immune infiltrate. Moreover, these lesions have a capacity for metastasis and as such do not carry the uniformly favorable prognosis of melanoma in situ. In the aforementioned patient, we tested both the primary tumor and the metastasis for expression of CDH3 and MMP-10. The in situ phase of the primary melanoma *and* the metastasis expressed both RGP markers, supporting the hypothesis that the radial growth clone is present in the metastasis and therefore gave rise to the metastasis (Fig. 4 and Color Plate 41). Whether expression of the RGP gene set contributes to the metastatic progression of melanoma is currently under investigation in murine models.

We then performed gene expression profiling of 19 metastatic melanomas from lymph node or subcutaneous tissue, determined to be positive by fine needle aspiration and subsequently analyzed by unsupervised SAM analysis. This revealed two molecular subtypes of melanoma metastasis (Fig. 5 and Color Plate 42): Type I metastasis in which 25% had RGP signatures, and all of these patients have since died; Type II metastasis in which 75% had VGP signatures

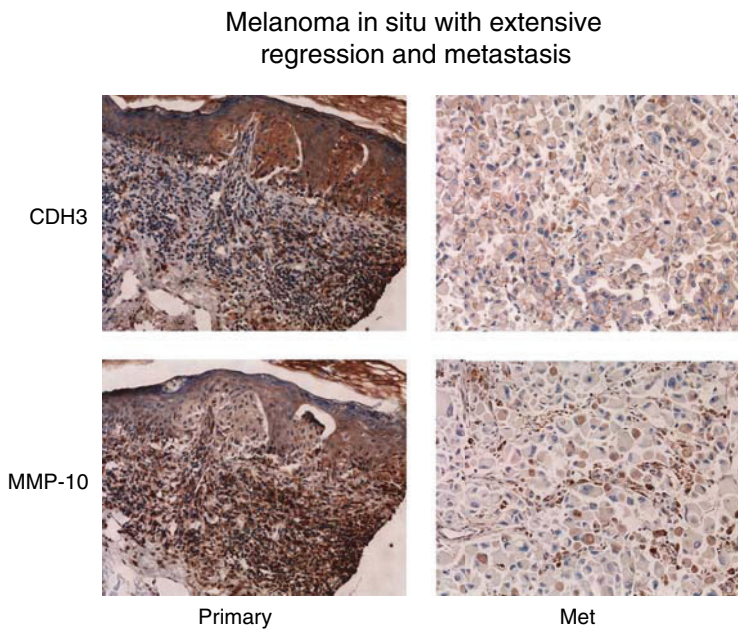

Fig. 4. Immunohistochemical staining for a patient with a melanoma in situ with extensive regression. The radial growth phase markers, CDH3 and MMP-10, were detected in both the primary melanoma and in the metastasis. (*see* Color Plate 41)

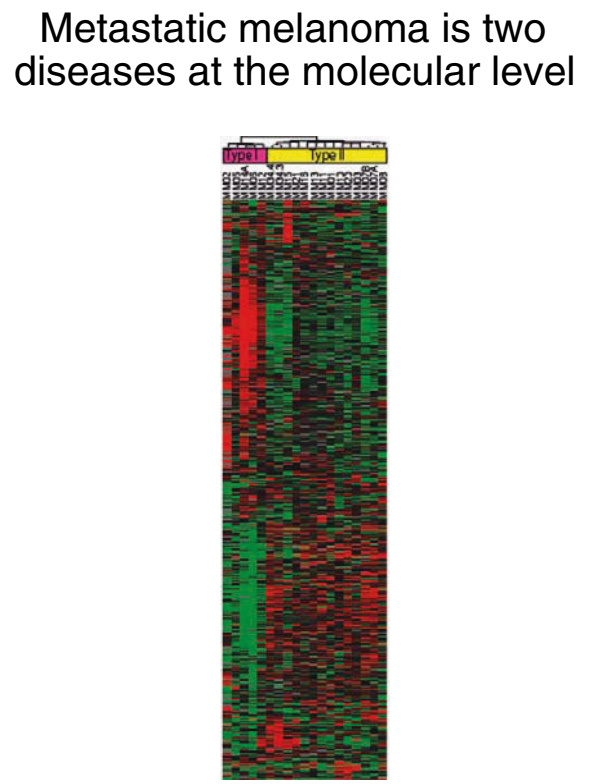

Fig. 5. Unsupervised hierarchial clustering of metastatic melanoma defines subtypes I and II. (*see* Color Plate 42)

and one third of these patients are alive. Thus, a subset of metastatic cases displayed RGP signatures at the molecular level. In a single patient in whom both primary tumor and lymph node metastasis were available for profiling analysis, the primary (a nodular melanoma) exhibited the VGP pattern at the molecular level, whereas the lymph node metastasis displayed the RGP gene signature. The profound implication is that the RGP of melanoma contains the molecular program for metastasis as this gene set was recapitulated in some of these metastatic cases.

Together, the loss of both adhesion and extracellular matrix proteins phenotypically changes the melanoma cell's adhesive properties and may allow the melanoma to leave the epidermis and undergo dermal invasion. However, the continued expression of these genes in the metastases suggests that the RGP clone is the clone that metastasized in a subset of melanomas, and that expression of these genes may contribute to or be required for the metastatic progression of melanomas from the RGP.

3.3.1. Distinguishing a Benign Nevus from a Primary Melanoma

Unsupervised hierarchical cluster analysis was able to distinguish between benign nevi and primary melanoma as evidenced by significant gains and losses of gene expression (Fig. 6 and Color Plate 43). Genes which were upregulated included osteopontin-1 (SPP-1), CXCL1, and RAB32, all having roles in melanoma progression. Genes downregulated were those with potential tumor suppressor activity: WIF1, ECM2, and SLIT3. Of all the genes in this analysis, osteopontin-1 showed the highest ratio of overexpression in primary melanomas when compared with nevi, suggesting a role as a biomarker in melanoma.

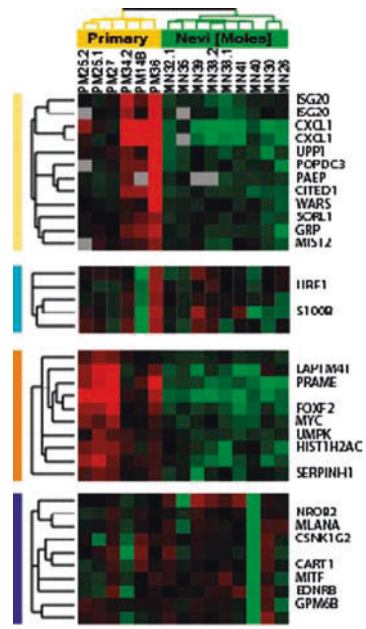

Fig. 6. Microanalysis is able to distinguish nevi from melanoma. (*see* Color Plate 43)

3.4. Osteopontin as a Novel Molecular Prognostic Marker for Melanoma

Osteopontin (OPN) is a secreted phosphoprotein encoded by the SPP1 gene, with a specific integrin-binding amino acid sequence (arginine–glycine–aspartic acid). Integrins are the major family of proteins expressed on the cell surface that mediate anchoring to the ECM and are involved in cell–cell adhesion. The interaction of OPN with members of the integrin family has been shown to trigger events necessary for tumor invasion including cell attachment and migration *(16)*. OPN expression is implicated in the progression of several human cancers including lung, breast, prostate, colon, duodenum, stomach, bladder, ovary, thyroid, pancreas, and melanoma *(9,40,36)*. In their model of tumor progression of squamous cell carcinoma in nude mice, Crawford et al. demonstrated that tumor-derived OPN can inhibit macrophage function and enhance the growth and survival of metastasis *(12)*. Malignant clones produced OPN in an autocrine fashion, inhibiting macrophage activation, giving the OPN-producing clones a selection advantage, and allowing them to evade immune surveillance and metastasize. Clones that did not express OPN were eliminated. The induction of OPN in several distinct human malignancies suggests a basic and important role of OPN in tumorigenesis.

The association of OPN and increasing invasiveness of melanoma was first described in a comprehensive gene profiling analysis of 31 cutaneous melanomas *(7)*. The identified gene clusters were validated by in vitro studies, showing that lesions can be classified by gene expression profiling. In a recent study, the prognostic utility of OPN as an independent molecular prognostic marker was determined using tissue microarrays containing melanomas from 345 patients (Rangel J, Nosrati M, et al., in press). There was a strong correlation between increasing staining of OPN and several histologic parameters including tumor thickness, Clark's level of invasion and mitotic index. High OPN expression correlated with an increased risk of relapse,

reduced RFS, and decreased disease-specific survival (DSS). Using univariate logistic regression, OPN was significantly associated with SLN metastasis ($p = 0.0009$) and SLN tumor burden ($p = 0.0025$). The predictive value of OPN was assessed using multivariate logistic regression analysis with current prognostic factors (tumor thickness, ulceration, Clark's level, age, and site). After patient age ($p = 0.0001$) and tumor thickness ($p = 0.0014$), OPN was found to independently predict SLN metastasis ($p = 0.0062$). These data strongly support a role of OPN in melanoma progression. OPN overexpression in metastatic melanoma has been shown in several other profiling studies *(25,45,51)*

3.4.1. Perform a Multiclass Analysis Comparing nevi, Primary, and Metastatic Lesions

The final objective of the profiling study was to identify biomarkers with the ability to separate nevi from primary melanoma and also to distinguish metastatic tissue samples from other tissues. The melanocytic lineage marker, S100 and Melan-A were unable to distinguish nevi from melanoma. However, several genes were overexpressed in metastatic lesions including NCOA3 (nuclear receptor coactivator receptor protein 3).

3.5. Molecular Marker NCOA3 Overexpressed in Metastatic Melanoma

NCOA3 (or AIB1—amplified in breast cancer 1) is an oncogenic member of the SRC (steroid receptor coactivator) gene family. In addition to having a role in female reproductive development, NCOA3 also has a role in cell growth, differentiation, migration, lymphopoesis, and cytokine signaling *(29)*. NCOA3 is required for normal mammary tissue development and is overexpressed and amplified in a large percentage of breast cancers *(1,5,30,38)*. It has also been shown to be overexpressed in ovarian *(1)* and endometrial cancer *(22)*. Recent microarray analyses of specimens of prostate cancer revealed (by immunohistochemistry) that overexpression of NCOA3 correlated with earlier recurrences *(50)*. Comparative genomic hybridization (CGH) was performed on a number of primary human gastric tumors and revealed amplification of NCOA3 *(42)*. More importantly, this was associated with a poorer prognosis and metastatic disease (lymph node, liver) compared to those without amplification. In another study, CGH of several pancreatic cancer cell lines demonstrated amplification (six of nine cell lines) of the NCOA3 gene *(21)*. There were multiple chromosomal aberrations clustered on several chromosomes including gains of chromosome 20q. These findings support the notion that NCOA3 has novel tumorigenic functions outside of its known steroid receptor activator role.

As demonstrated by recent cDNA microarray analysis, NCOA3 was differentially expressed in metastatic versus unrelated primary melanomas *(24)*. Since metastases are derived from primary tumors, those primary melanomas exhibiting higher levels of NCOA3 expression would be expected to have a higher risk of relapse and death due to melanoma. As a result, NCOA3 was postulated to act as a molecular prognostic marker for melanoma. Based on these findings, for the first time, the prognostic significance of NCOA3 overexpression in melanoma was evaluated using tissue microarrays in a cohort of 343 primary melanomas *(37)*. High NCOA3 immunostaining correlated significantly with melanoma recurrence, reduced RFS and decreased DSS. The prognostic power of NCOA3 was compared to current AJCC prognostic factors by multivariate Cox regression. NCOA3 was found to be an independent predictor of RFS and outperformed all factors for DSS. Increasing NCOA3 staining correlated significantly with nodal metastasis and tumor burden. Patients with a staining score of 0 had a 7.1% prevalence of SLN metastasis which increased to 38.3% in patients with a score of 3. The mean number of nodes involved was 0.07 in patients with staining score of 0 and 0.57 nodes with a staining score of 3. Interestingly, expression of NCOA3 did not significantly correlate with histologic subtype of melanoma. Of the nine DMMs, only one exhibited intense staining. This is

an interesting finding since DMMs infrequently metastasize to lymph nodes. However, routine SLN biopsy of DMM > 1 mm (or <1 mm with ulceration or Clark's Level IV invasion) remains within the current scope of practice although its proper use continues to be debated.

4. MOLECULAR MARKERS AND SENTINEL LYMPH NODE BIOPSY

What if we could predict that a patient would develop nodal metastasis based on staining of the primary melanoma? Intriguingly, the metastases examined in the gene expression profiling study *(24)* that overexpressed NCOA3 were primarily lymph node metastases suggesting the potential importance of NCOA3 expression to melanoma lymph node metastasis. The other molecular marker that appears to accurately predict SLN metastasis is OPN. In patients with OPN staining scoring of 0, SLN metastasis was observed in 8.8% of patients, however in patients scoring of 1, 2, or 3 this increased to 32.9%. As with NCOA3, OPN scoring correlated with SLN tumor burden: score of 0, the mean number of nodes involved was 0.15; score 1, 2, or 3 the mean number of nodes was 0.53. Increasing staining of both OPN and NCOA3 predicted SLN disease and tumor burden.

Two groups of patients in which the role of SLN biopsy remain controversial are those with DMM and those patients with thin melanomas. A recent retrospective analysis of patients with DMM *(13)* undergoing either SLN biopsy or a radical neck dissection, revealed 1/18 patients with nodal disease at the time of staging, supporting the infrequent reporting of metastasis in this subtype of melanoma. Although purely DMMs appear to have a better prognosis based on tumor thickness when compared with conventional melanoma, metastatic disease does occur.

The metastatic potential of thin (<1 mm) melanomas was evaluated in 43 metastasizing thin melanomas including two melanoma in situ lesions *(23)*. One of the in situ lesions had extensive regression however the other did not, emphasizing the fact that Clark's Level I melanomas may not be (rather than or not) biologically immune from metastatic potential.

In thin melanomas, there is no reliable marker to predict metastatic potential and current guidelines advocate SLN biopsy in melanomas that are >1.0 mm in thickness or <1.0 mm but with ulceration or Clark's level IV of invasion. However, melanomas < 1 mm without high-risk features do metastasize. More importantly, we now know that RGP gene signatures, representing the minimally invasive phase of melanoma, are found in a subset of metastatic melanomas *(24)*.

The identification of novel molecular markers such as NCOA3 and OPN to predict SLN disease suggests its potential utility in identifying candidates to undergo this procedure.

5. IMPLICATIONS AND FUTURE DIRECTION

At this time, mounting evidence supports the notion that the radial growth phase of melanoma contains genes with the potential to allow for metastatic disease. We now postulate that a primary melanoma with only RGP signatures may proceed to the metastatic phenotype, bypassing an obvious vertical component (Fig. 7 and Color Plate 44).

Ultimately, prognostic factors should give us a sense of the behavior of an individual's melanoma that will allow a customized approach to management. However, the heterogeneous nature of melanoma at the clinical and histologic level, make predicting which patients will develop metastatic disease difficult. In the current era of molecular biology, recent advances in biomarker development in melanoma strongly suggest that molecular markers will allow for better patient selection for both targeted therapies and SLN biopsy. Gene profiling analyses will more clearly define melanomas and determine the role of these profiles in different clinical outcomes. While it is important for the biomarkers discussed in this chapter to undergo further

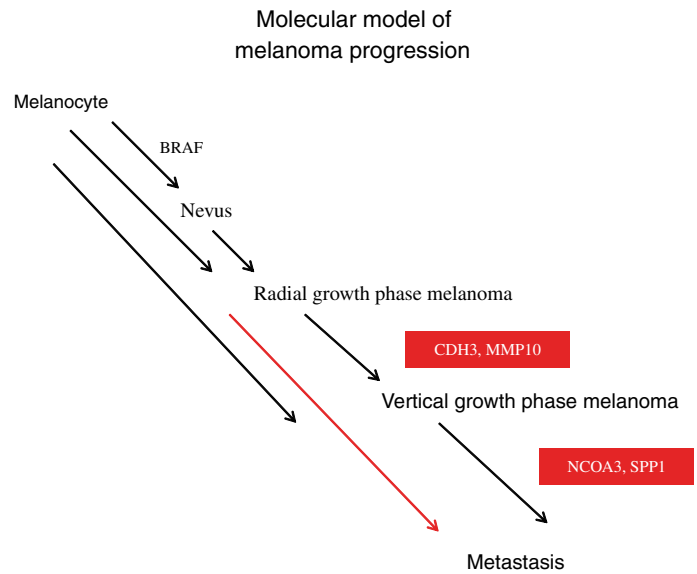

Fig. 7. Primary melanoma to metastasis: radial growth phase melanomas have the potential to metastasize and the development of a vertical growth phase may not be a prerequisite for the development of metastatic disease. (*see* Color Plate 44)

validation, the powerful and independent prognostic impact of these markers after inclusion of classical prognostic factors comprising the current AJCC classification strongly suggests the ushering of the molecular era into the prognostic assessment of melanoma patients.

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XII Promising New Treatments for Solid Tumors

42 Targeted Therapy for Breast Cancer: A Focus on HER2/neu and Antiangiogenic Therapy

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CONTENTS

ABSTRACT

Breast cancer is the most common female cancer in the USA and is the main cause of death in women aged 45–55 years. Despite advances in both diagnosis and treatment, an estimated 182,460 new cases (26% of all cancers) and 40,480 deaths are expected in 2008. One area of rapid evolution is the treatment of patients with HER2/neu overexpressing breast tumors, who comprise 25–30% of all breast cancers in the early stage setting. In conjunction with chemotherapy, trastuzumab has shown efficacy in both adjuvant and metastatic settings with improved response rate, time to progression, and overall survival. Lapatinib, a multitargeted tyrosine kinase inhibitor (TKI), is approved for trastuzumab-resistant disease in combination with capecitabine; multiple studies are evaluating lapatinib in combination with other chemotherapy agents as well as targeted biologic therapy. Additional anti-HER2/neu-targeted agents under study include immunotoxins, alternate antibodies, new TKIs, and new targeted therapies such as heat shock protein inhibitors. Antiangiogenic therapy is a second successful targeted biologic therapy for the treatment of breast cancer. Bevacizumab is now approved in combination with paclitaxel to treat advanced disease, and ongoing trials are investigating bevacizumab alone or in combination in both the early- and late-stage settings. Multiple other antiangiogenic agents are in clinical trials. This chapter will review current clinical data on HER2 and antiangiogenic biologic therapies in the treatment of breast cancer.

Key Words: metastatic breast cancer; HER2/neu; trastuzumab; lapatinib; bevacizumab; sunitinib; antiangiogenic; targeted therapy

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1. INTRODUCTION

Breast cancer is the most common female cancer in the USA and is the main cause of death in women aged 45–55 years *(1)*. Risk factors for breast cancer include genetic mutations such as BRCA 1 and 2, female gender, age, family history, prolonged exposure to estrogen (e.g. early menarche, late menopause, extended hormone replacement therapy), previous exposure to chest irradiation, and benign proliferative breast disease with atypia *(1,2)*. Despite advances in both diagnosis and treatment, in the USA an estimated 182,460 new cases of breast cancer (26% of all cancers) and 40,480 deaths are expected in 2008 *(1)*. Improvements in outcome will require a greater understanding of the biologic and genetic factors that drive tumor growth and resistance. Currently, treatment decisions are based on a number of tumor-related factors including hormone receptor status, the presence or absence of HER2/neu gene amplification or protein overexpression, tumor grade, as well as extent of disease. The limitations of this approach are demonstrated by data indicating marked variation in outcome among breast tumors based on gene expression arrays *(3,4)*. Identification of the factors that drive tumor growth and invasiveness is critical for the development of effective treatment, and has already led to therapy targeted to tumor biology. However, the limitations of this approach are related to identification of factors that predict individual response to treatment.

2. TARGETING HER2/NEU

Recent gene expression studies have demonstrated significant heterogeneity among breast tumors that have been closely associated with outcome *(5–7)*. Initial identification of the HER2/neu oncogene by Slamon in 1987 *(8)* led to successful development of the humanized antibody trastuzumab. By targeting the 25–30% of breast tumors found to overexpress the HER2/neu oncoprotein or have HER2/neu gene amplification, remarkable clinical benefits have been demonstrated *(9,10)*. Trastuzumab was initially tested in combination with chemotherapy for the treatment of metastatic breast cancer, with response rates generally over 50% *(11)*. Treatment with weekly trastuzumab and paclitaxel resulted in an overall response rate of 67–81%, and was significantly higher in patients known to have HER2/neu-positive disease *(12)*. In the pivotal phase III trial, 234 women with chemotherapy naïve metastatic disease with HER2/neu-positive disease by immunohistochemistry (IHC), or gene amplification by fluorescent in situ hybridization (FISH) were randomized to chemotherapy (doxorubucin plus cyclophosphomide (AC), or paclitaxel (based on prior exposure to anthracycline or not) with or without trastuzumab *(9)*. Treatment with trastuzumab resulted in a longer time to progression (7.4 vs. 4.6 months with chemotherapy only), higher response rate (50 vs. 32 %), and longer median overall survival (overall survival, 25.1 vs. 20.3 months) resulting in FDA approval of trastuzumab in combination with paclitaxel for the treatment of metastatic, HER2/neu-positive breast cancer. Patients treated with an anthracycline combined with trastuzumab experienced high rates of cardiac toxicity (27%) leading to the discontinuation of this combination in clinical practice, but otherwise treatment was well tolerated.

A subsequent randomized phase II trial evaluated docetaxel with or without trastuzumab in the first-line metastatic setting and showed similar results; 186 patients receiving both agents had an overall response rate of 61% compared to 34% in the docetaxel-alone group *(13)*. In addition, time to disease progression (11.7 vs. 6.1 months), and duration of response (11.7 vs. 5.7 months) were prolonged in trastuzumab-treated group. Although initial results demonstrated a survival benefit (median overall survival 31.2 vs. 22.7 months), on longer follow-up and with significant crossover of control arm patients to trastuzumab, this difference disappeared. Single-agent trastuzumab is also effective although with lower response rates, one trial treated 114 women with HER2/neu-positive metastatic disease in the first-line setting and reported an

objective response rate of 26% *(14)*. Multiple combinations of trastuzumab with chemotherapy have been tested in phase II clinical trials, all with modest response rates. Recent data suggest that trastuzumab could reverse at least some degree of resistance to hormone therapy in HER2/neu-positive disease. Two hundred and seven patients with hormone receptor-positive metastatic breast cancer that also overexpressed HER2/neu were randomized to the aromatase inhibitor anastrozole versus anastrozole plus trastuzumab *(15)*. Progression-free survival (PFS) was prolonged from 2.4 to 4.8 months ($p = 0.0016$) in the trastuzumab-treated arm; the partial response (PR) rate in the subset of patients evaluable for response was increased from 6.8 to 20.3% ($p = 0.018$). The short PFS even with combination therapy is somewhat discouraging, but clearly demonstrates a role for trastuzumab in reversing resistance to hormone therapy.

The addition of trastuzumab to standard adjuvant chemotherapy has resulted in marked improvements in outcome in four large randomized trials. A joint analysis of the National Surgical and Bowel Project (NSABP) B-31 and North Central Cancer Treatment Group (NCCTG) N9831 trials included 3,351 patients with HER2/neu-positive, axillary node-positive (and a small number of patients with high risk, node-negative disease) breast cancer who were randomized to four cycles of AC every 3 weeks followed by four cycles of paclitaxel (given every 3 weeks or weekly) with or without concurrent trastuzumab, with trastuzumab continued for a total duration of 1 year *(10)*. A third arm of the NCCTG trial evaluated trastuzumab sequential to chemotherapy, but has not yet reported final data. At a median follow-up of 2 years, there was a 33% reduction in the risk of death ($p = 0.015$) and a 12% absolute difference in disease-free survival (DFS) (87 vs. 75%, $p < 0.0001$) between the trastuzumab and control groups, with the 3-year cumulative incidence of class III or IV congestive heart failure or death from cardiac causes in the trastuzumab group ranging from 2.9 to 4.1%. BCIRG 006 randomized 3,222 women with HER2neu-positive high-risk node-negative or node-positive disease to three treatment arms: four cycles of AC followed by four cycles of docetaxel (AC-T); AC-T combined with trastuzumab during docetaxel and continued for nine additional months (AC-TH); or six cycles of docetaxel, carboplatin, and trastuzumab (TCH) followed by 9 months of trastuzumab alone *(16)*. The second interim analysis was performed at a median follow-up of 36 months and demonstrated a similar improvement in DFS in both trastuzumab arms (AC-TH 83%, TCH 82%) compared to the AC-T arm (77%) for an absolute improvement at year 4 of 6 and 5%, respectively. Similarly, overall survival was significantly improved in patients treated with trastuzumab (AC-TH 92%, TCH 91%) compared to AC-T (86%). In this study, cardiac decline (relative left ventricular ejection fraction [LVEF] decrease from baseline >10%) varied depending on the regimen and use of anthracycline (18% AC-TH: 18%, AC-T: 10%, TCH: 9%). The three-arm HERA trial randomized 5,102 women with HER2/neu-positive node-positive or high-risk node-negative breast cancer to 1 or 2 years of trastuzumab versus no additional treatment after completion of adjuvant or neoadjuvant chemotherapy *(17)*. The 2-year data have not yet been analyzed; data are available from the comparison of 1 year of trastuzumab versus chemotherapy alone. At a median follow-up of 23.5 months, 1 year of trastuzumab improved DFS by 6.3% (hazard ratio [HR] 0.64; $p < 0.0001$) and overall survival by 2.7% (0.66; $p = 0.0115$); symptomatic congestive heart failure was observed in 2.2% of the population. *(18)* On the basis of these data, trastuzumab was approved in combination with chemotherapy for treatment of early stage, HER2/neu overexpressing breast cancer, The very small FinHer study enrolled a subset of 232 patients with HER2/neu-positive disease who were randomized on a larger chemotherapy study (vinorelbine vs. docetaxel followed by fluorouracil, epirubicin, cyclophosphamide), and then were randomized to trastuzumab for 9 weeks given concurrently with vinorelbine or docetaxel *(19)*. At a median follow-up of 3 years, DFS was 89% in trastuzumab group and 78% in chemotherapy-alone group ($p = 0.01$). There were no cases of decreased ejection fraction or heart failure.

3. LAPATINIB

A number of new agents targeting HER2/neu are under investigation and one novel agent is approved for the treatment of advanced disease. Oral tyrosine kinase inhibitors (TKIs) are small molecules that inhibit phosphorylation of the tyrosine kinase on certain transmembrane receptors, consequently blocking downstream signaling. TKIs have the theoretical ability to overcome some mechanisms of resistance; e.g., mutations or degradation of the receptor from the cell surface that would reduce antibody efficacy, heterodimerization of HER2 with other receptors, and mutations in partner molecules. In addition, small molecules could potentially cross the blood brain barrier, a difficult to treat area not reached by standard chemotherapy agents.

Lapatinib is a multitargeted TKI that blocks the tyrosine kinase activity of HER2 homodimers as well as heterodimers. As monotherapy for untreated HER2/neu-positive locally advanced or metastatic breast cancer in 138 patients, treatment with lapatinib resulted in an overall response rate of 24% with PFS at 4 and 6 months of 63% and 43%, respectively *(20)*, including one response in brain. The most common side effects (e.g., diarrhea, rash, pruritis, and nausea) were grade 1 or 2; treatment was well tolerated. In 140 patients with chemotherapy and trastuzumab refractory HER2/neu-positive disease, lapatinib as a single agent had modest clinical activity (response rate 4.3 and 1.4% by investigator and independent review, respectively) *(21)*. In 89 patients with HER2/neu-negative and chemotherapy refractory disease, no objective response was seen.

Currently, lapatinib is approved in combination with capecitabine for the treatment of trastuzumab-resistant advanced breast cancer. EGF100151 was a phase III trial that randomized patients with HER2/neu-positive and trastuzumab pretreated advanced breast cancer to capecitabine with or without lapatinib at 1,250 mg a day *(22)*. Mean time to progression was 36.7 weeks in the combination arm ($n = 160$) versus 19.1 weeks in the capecitabine only group ($n = 161$, $p = 0.00004$). Both median PFS (36.7 weeks vs. 17.9 weeks, $p = 0.00001$) and response rate (22 vs. 14%) were superior in the combination arm; most of the responses were partial remissions (21%). The addition of lapatinib was well tolerated with primary toxicities of modest or mild diarrhea, nausea, and rash. There was no significant difference in the rate of asymptomatic decrease in ejection fraction, and other trials have similarly failed to uncover significant cardiac toxicity associated with lapatinib. There were numerically less brain metastases in patients receiving lapatinib (13 vs. 4, $p = 0.045$) *(22)*, suggesting that lapatinib crosses the blood brain barrier and may be an effective prevention or treatment strategy for this challenging site. These encouraging data led to a trial evaluating lapatinib in patients with HER2/neu-positive brain metastases progressing following trastuzumab and radiation therapy *(23)*. A toatal of 241 patients were treated with lapatinib 750 mg twice a day and with option to continue on lapatinib and capecitabine therapy upon progression of disease. Six percent of 117 evaluable patients had a PR, and 42% had stable disease. Responding patients had a longer PFS, at 25.3 versus 15.3 weeks. Forty patients went on to the extension arm lapatinib and capecitabine; with 40% of patients had a greater than 20% reduction in brain metastases, and 20% showed a greater than 50% reduction.

Other trials are evaluating a variety of combinations with lapatinib. A phase III trial treated 579 patients with chemotherapy and trastuzumab naïve advanced breast cancer with every 3-week paclitaxel with or without lapatinib *(24)*. Diarrhea was more common in the lapatinib-treated patients (8 vs. <1%), as were all grades of rash and mucositis. There was an increase in toxic deaths with combination therapy (2.7 vs. 0.6%), thought to be due to the initial lack of experience with managing diarrhea, and a pharmacokinetic interaction between lapatinib and paclitaxel that results in ~20% increase in AUC for both drugs. In the entire cohort, lapatinib

improved investigator assessed response rate, but not PFS. In contrast, in the 15–19% of patients with HER2-positive disease, lapatinib improved TTP (7.9 vs. 5.2 months, $p = 0.007$, HR 0.56) and response rate (60 vs. 36%, $p = 0.027$, OR 2.9), with a trend toward improved overall survival. A second phase III trial comparing the aromatase inhibitor letrozole with or without lapatinib in patients with hormone receptor-positive advanced disease has completed accrual and should report initial data in late 2008 or 2009. The possibility of overcoming resistance by blocking HER2 signaling via multiple mechanisms is an intriguing concept tested in a recently presented phase III trial that randomized 296 patients with HER2/neu-positive disease refractory to prior chemotherapy (anthracycline and taxane) and trastuzumab therapy to lapatinib (1,500 mg/day) or lapatinib (1,000 mg/day) combined with tratuzumab *(25)*. Crossover from the lapatinib to the combination was allowed. The PFS was significantly longer in patients receiving the combination therapy at 12 versus 8 weeks ($p = 0.0$–08), with no significant increase in toxicity. This study provides supportive data for the combination arm of the adjuvant ALTTO trial described below. Multiple phase II trials testing a variety of combinations are ongoing.

Based on the encouraging results of the adjuvant trastuzumab trials, and results from the lapatinib trials, a large cooperative group adjuvant trial (ALTTO) has been launched between the US Breast Intergroup and the Breast International Group. This study (BIG 2.06/NCCTG N063D) will randomize 8,000 women with Her2/neu-positive early-stage breast cancer who have completed surgery and (neo)adjuvant anthracycline-based chemotherapy to one of four treatment arms (with or without 12 doses of weekly paclitaxel per physician preference): trastuzumab for 1 year, lapatinib for 1 year, trastuzumab for 3 months followed by a 6-week break and then lapatinib for 7.5 months, or trastuzumab every 3 weeks in combination with lapatinib for 1 year along with standard radiation therapy and endocrine therapy as indicated. A recent phase II trial has demonstrated significant toxicity including a 20% rate of grade 3 diarrhea in patients treated with the combination of weekly paclitaxel, trastuzumab, and lapatinib at 1,000 mg a day *(26)*. Based on these data, the dose of lapatinib in the ALTTO trial has been reduced to 750 mg a day during concomitant trastuzumab and paclitaxel, and then is increased again to full dose at the end of chemotherapy. Two multicenter neoadjuvant trials are evaluating taxane chemotherapy with single-agent lapatinib and trastuzumab versus the combination with correlative endpoints.

4. NOVEL HER2-DIRECTED THERAPY

Many additional HER2/neu-directed therapies are under evaluation including antibodies, immunotoxins, small molecules, and vaccines. A phase II trial treated 102 patients with or without prior treatment with trastuzumab with HKI-272, an irreversible pan-HER-targeted TKI *(27)*. Response rates ranged from 22% in patients with previous trastuzumab treatment, to 53% in trastuzumab naïve disease. 26% of patients experienced grade 3/4 diarrhea; combination studies are planned. Pertuzumab is a humanized monoclonal antibody that targets the extracellular portion of the HER2/neu receptor to prevent both homodimerization as well as heterodimerization with other receptors, therefore blocking multiple HER signaling pathways. A total of 61 patients with trastuzumab-resistant advanced breast cancer were treated with the combination of pertuzumab and trastuzumab in a phase I study. The combination was well tolerated, and in 33 evaluable patients the overall response rate was 18%. Updated data will be presented in 2008, and additional trials are planned or ongoing.

Trastuzumab–DM1 (T-DM1) is a HER2 antibody–drug conjugate. DM1 is a highly potent antimicrotubule agent, and T-DM1 binds to HER2 to deliver the chemotherapeutic agent directly to the cancer cell, minimizing systemic exposure. A phase I dose escalation trial in

24 patients demonstrated clinical activity with six objective responses in this heavily pretreated group with disease resistant to trastuzumab; the dose limiting toxicity was reversible thrombocytopenia *(28)*. A phase II trial is ongoing with the future plan to compare trastuzumab and chemotherapy to T-DM1 as treatment for advanced, HER2-positive disease. 17-allylamino, 17-demethoxygeldanamycin (17-AAG) is an ansamycin antibiotic inhibitor that binds to the ADP/ATP switch site of heat shock protein-90 (HSP-90). HSP-90 *(29)* acts as a molecular chaperone protein and is important for maturation and stabilization of key signaling proteins such as HER2, raf kinase, AKT, ER, PR, bcr-abl, mutant p53, and mutant b-raf. Inhibition of HSP-90 has the potential to inhibit multiple oncogenic proteins and pathways. In the trastuzumab-sensitive SkBr-3 cell line, 17-AAG treatment for 24 h resulted in complete apoptosis *(30)*.

Studies with 17-AAG have been complicated by toxicity and solubility issues; the first generation of this agent to be tested in clinical trials in breast cancer was solubilized in Cremophor *(31)*. A recently reported phase I study examined whether the HSP-90 inhibitor tanespimycin (17-AAG; KOS-953) could be administered safely in combination with trastuzumab at a dose that inhibits HSP-90 function in vivo in lymphocytes *(32)*. Tanespimycin was given at four dose levels to 25 patients with advanced HER2-positive breast cancer. Toxicity included thrombocytopenia, fatigue, and gastrointestinal side effects including elevated transaminases. All dose levels appeared to inhibit HSP-90 in pharmacodynamic testing, and antitumor activity was noted with one patient experiencing a partial remission, and eight patients with stable disease or a minor response. Additional studies are ongoing testing this and other similar agents that are water soluble.

5. TARGETING HER2 AND THE VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR

Data from human breast cancer lysates have demonstrated a significant positive association between HER-2/neu and vascular endothelial growth factor (VEGF) expression, with a poorer prognosis in tumors that overexpress both HER2/neu and VEGF *(33)*. These studies provided the background for a phase I/II trial testing the combination of trastuzumab with bevacizumab, an antibody to VEGF. In the 37 evaluable patients reported to date from the phase II trial, the overall response rate was 54% (3% complete and 51% partial); cardiac toxicity was observed at a low rate *(34)*. The positive results from this trial have led to two ongoing phase III randomized trials testing the addition of bevacizumab to a combination of chemotherapy and trastuzumab; the BETH trial in the adjuvant setting, and the ECOG 1103 trial in the first-line metastatic setting.

Combinations with oral TKIs have also tested this concept. The combination of lapatinib and bevacizumab is being evaluated in an ongoing phase II clinical trial; preliminary data were presented at ASCO in 2008 in the first 32 patients demonstrating a partial response rate of 13% and a clinical benefit rate of 35% in largely trastuzumab refractory disease *(35)*. PFS was 63%, and there were no unexpected toxicity signals. Updated data will be presented in late 2008. Lapatinib alone (1,500 mg/day) is being compared to the combination of lapatinib (1,000 mg/day) and the oral vascular endothelial growth factor receptor (VEGFR)-targeted TKI pazopanib in a randomized phase II trial in 141 patients with trastuzumab and chemotherapy naïve metastatic HER2-positive breast cancer *(36)*. The endpoint is 12-week PFS, to allow nonresponding patients to quickly receive trastuzumab. Two doses of pazopanib are being tested, preliminary results are available from the lower dose arm (400 mg/day) suggesting a marked improvement in PFS at 12 weeks in the combination arm (63 vs. 84%, $p = 0.0091$). This corresponded to a significant

improvement in response rate as well, 27.8 vs. 44.9%. Treatment was generally well tolerated, with four patients experiencing a drop in LVEF requiring cessation of therapy. The higher dose trial is ongoing, and final analysis of this dataset will be provided in the future.

6. ANTIANGIOGENIC THERAPY

Hypoxia in tumors plays a key role in stimulating new vessel growth via the VEGF pathway and preclinical studies have demonstrated additive effects when bevacizumab is combined with taxanes (among other agents), making this an attractive target for antitumor therapy. Bevacizumab demonstrated low-level activity in the metastatic setting as a single agent with response rates of about 9% *(37)*, and a phase III trial in anthracycline and taxane pretreated disease in combination with capecitabine demonstrated an improvement in response, but no difference in PFS *(38)*. In the first-line setting, ECOG 2100 randomized 680 patients with metastatic breast cancer to paclitaxel (90 mg/m^2 given on days 1, 8, and 15 of 28-day cycle) with or without bevacizumab (10 mg/kg given on days 1 and 15) *(39)*. Bevacizumab improved response rate (36.9 vs. 21.2%, HR 0.60; $p < 0.001$) and PFS (5.9 vs. 11.8 months, $p < 0.001$), but the difference in overall survival was not significant ($p = 0.16$). More patients experienced grade 3/4 side effects in the combination arm including hypertension (14.8 vs. 0%, $p < 0.001$), proteinuria (3.6 vs. 0%, $p < 0.001$), headache (2.2 vs. 0%, $p = 0.008$), and infection (9.3 vs. 2.9%, $p < 0.001$). These data, combined with preliminary data comparing docetaxel alone or with bevacizumab in two different doses, led to accelerated approval of bevacizumab in combination with paclitaxel as first-line therapy for advanced breast cancer.

Bevacizumab is now being tested in the adjuvant and neoadjuvant settings. ECOG 5103 is currently randomizing patients with early stage disease to standard anthracycline- and taxane-containing chemotherapy with or without bevacizumab. A cooperative group trial will evaluate bevacizumab in combination with chemotherapy as neoadjuvant treatment of triple negative breast cancer, and a phase III trial is evaluating the combination of bevacizumab with hormone therapy compared to hormone therapy alone for advanced disease.

In addition to bevacizumab, many other antiangiogenic agents are in development or in clinical trials. Among the multiple oral TKIs, differential target specificity and toxicity has been found. For example, sunitinib blocks VEGFR as well as the platelet derived growth factor receptor (PDGFR) but axitinib is more specific to the VEGFR. Clearly this results in differences in observed toxicity such as hypertension, bone marrow suppression, and oral pain; differences in antitumor effects remain to be elucidated. Sunitinib has demonstrated modest single-agent activity in advanced heavily pretreated breast cancer (response rate of 11%) *(40)*, and is being actively studied in combination with both chemotherapy and hormonal therapy. Other anti-angiogenic TKIs including axitinib, pazopanib, sorafenib, vandetinib, and others are being studied either in combination with chemotherapy or hormone therapy in the metastatic setting.

Current investigation is focusing on developing new targets as well as understanding factors that determine response and resistance. In order to effectively use antiangiogenic and other novel targeted agents, it will be critical to identify predictors of response although this has to date been elusive. Rational combinations of novel biologic agents to overcome pathways of resistance is also an intriguing approach with encouraging preclinical data. Examples in recent or current clinical trials include the combination of antiangiogenic therapy with HER2-directed agents (trastuzumab and bevacizumab, lapatinib and bevacizumab, lapatinib and pazopanib, etc., described above), and combining various targeted agents with hormone therapy (letrozole or tamoxifen with bevacizumab [CALGB 40503], fulvestrant and lapatinib [CALGB 40302], and second-line hormone therapy with various antiangiogenic agents). Current and future trials will focus on breast cancer subsets with therapy directed toward unique biology.

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43 Advances in the Treatment of Colorectal Cancer: Targeting Receptors of Disease

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CONTENTS

ABSTRACT

The successful targeting of angiogenesis and epidermal growth factor receptor (EGFR) pathway has impacted the care of advanced colorectal cancer (CRC) patients. Bevacizumab was the first antiangiogenic agent to show survival benefit in first-line setting and further trials suggested similar efficacy in the second-line and maintenance settings. Cetuximab is an anti-EGFR monoclonal antibody that improved clinical outcomes for irinotecan-refractory advanced CRC patients and its role in first-line setting is under investigation. Panitumumab is a humanized anti-EGFR monoclonal antibody shown to improve the survival of treatment-refractory advanced CRC patients but failed to achieve similar benefits in first-line setting. Current research effort in CRC therapy focuses on developing the optimal approaches to integrate these biological agents into clinical care.

Key Words: colorectal cancer; bevacizumab; cetuximab; panitumumab; kras

1. INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the USA and an estimated 153,760 new cases will be diagnosed in 2007 *(1)*. Multidisciplinary approach is the cornerstone in the management of CRC patients. Fluorouracil-based regimens form the backbone of contem-

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Table 1
Contemporary Fluorouracil-Containing Cytotoxic Regimens in Advanced Colorectal Cancer

FOLFOX *(55)*	Infusional fluorouracil, leucovorin, oxaliplatin, repeat every 2 weeks
FOLFIRI *(56, 57)*	Infusional fluorouracil, leucovorin, irinotecan, repeat every 2 weeks
IFL (Saltz) *(58)*	Bolus fluorouracil, leucovorin, irinotecan weekly for 4 weeks every 6 weeks
5FU/LV (Mayo Regimen) *(59)*	Bolus fluorouracil daily for 5 days, repeat every 28 days
5FU/LV (Roswell Park Regimen) *(60)*	Bolus weekly fluorouracil for 6 out of 8 weeks
deGramont (infusional) *(61)*	Bolus fluorouracil followed by continuous infusion over 22 h for 2 days, repeat every 2 weeks

porary systemic chemotherapy in both adjuvant and metastatic settings (Table 1) *(2)*. The American Society of Clinical Oncology recommends adjuvant systemic chemotherapy for stage III colon cancer but not routine use in stage II patients *(3)*. In advanced or metastatic disease, the use of fluorouracil-based combination therapy has resulted in significant survival prolongation. This improvement was possible with the advent of active chemotherapeutic agents, including conventional cytotoxics, such as oxaliplatin and irinotecan, and novel targeted agents, such as bevacizumab, cetuximab, and panitumumab.

This chapter will discuss the latest advances in the development of targeted biological agents in CRC (Table 2). Angiogenesis and epidermal growth factor receptor pathways have been successfully exploited in CRC therapy. The biological agents targeting these pathways often have poor single-agent activity but become synergistic when administered in combination with cytotoxic agents.

Table 2
Summary of Novel Antiangiogenic and EGFR Agents in Advanced Colorectal Cancer

	Nature of drug	*Target(s)*	*Clinical use*
Anti-angiogenic			
Bevacizumab	Humanized monoclonal antibody	VEGF	FDA approved for first-line metastatic colorectal cancer setting with fluorouracil-containing regimens.
Sunitinib	Small-molecule kinase inhibitor	VEGFR-2, PDGFR, c-kit, FLT-3	Undergoing clinical evaluation in advanced colorectal cancer.
Anti-EGFR			
Cetuximab	Chimeric murine-human monoclonal antibody	EGFR	FDA approved for use as single agent or in combination with irinotecan in irinotecan-refractory metastatic colorectal cancer patients with EGFR-expressing tumor.
Panitumumab	Humanized monoclonal antibody	EGFR	FDA approved for treatment of patients with EGFR-expressing metastatic colorectal cancer refractory to oxaliplatin, irinotecan and fluoropyrimidine-containing regimens.

2. ANGIOGENESIS

Angiogenesis refers to the formation of new blood vessels from existing vasculature and is regulated by a number of pro- and antiangiogenic factors. Physiologically, the process is important for growth, reproduction and development but is dysregulated in neoplasm *(4)*. The pathological vasculature formed allows tumors to grow beyond 1–2 mm *(3)*, the physiological limit within which tumor growth can be supported by the diffusion of oxygen and nutrients. However, this vasculature is often leaky and dysfunctional in tumors, leading to increased interstitial pressure that impedes the delivery of both nutrients and chemotherapeutic agents *(5)*.

A number of molecules have been implicated as positive mediators of angiogenesis, including fibroblast growth factor, transforming growth factor-α/β, hepatocyte growth factor and tumor necrosis factor-α. Subsequently, vascular endothelial growth factor (VEGF)-A, commonly known as VEGF, signaling was found to represent a critical rate-limiting step in angiogenesis *(6)*. VEGF affects neovascularization through interacting with two transmembrane receptor tyrosine kinases: VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1, or KDE). VEGFR-2 is the main mediator of the actions of VEGF and is often overexpressed in tumor vasculatures. VEGFR-2 activation promotes endothelial cell proliferation, survival and migration. Much interest exists in targeting VEGF and VEGFR-2 in anticancer therapy. This can be accomplished by monoclonal antibody intercepting the ligands, such as bevacizumab, or small-molecule tyrosine kinase inhibitors, such as sunitinib.

2.1. Bevacizumab

Bevacizumab is a humanized recombinant monoclonal antibody that intercepts VEGF, thereby inhibit angiogenesis *(7)*. The agent was first approved in the USA for use in CRC following convincing data from a multicenter randomized trial. In this landmark phase III trial, 813 patients with newly diagnosed metastatic CRC were randomized to receive irinotecan, bolus fluorouracil, and leucovorin (IFL) plus placebo (control), and IFL plus bevacizumab (5 mg/kg) or fluorouracil and leucovorin (5FU/LV) plus bevacizumab (5 mg/kg) *(8)*. The 5FU/LV plus bevacizumab arm was discontinued later during planned interim analysis when the IFL plus bevacizumab arm proved to be safe. Compared to the control arm, the bevacizumab-containing arm demonstrated superior median survival (20.3 vs. 15.6 months) and response rate (44.8 vs. 34.8%). The patients receiving bevacizumab reported higher frequency of reversible hypertension and proteinuria. Serious but rare toxicities included wound dehiscence, thrombosis, and gastrointestinal perforation. Subsequently, bevacizumab is approved by the Food and Drug Administration (FDA) for use with fluorouracil-containing regimens in CRC in the first-line setting *(7)*.

The efficacy in CRC seems to be higher when bevacizumab is combined with infusional fluorouracil regimen in the BICC-C trial *(9)*. This multicenter randomized trial was initially designed to study the optimal fluoropyrimidine backbone for the addition of irinotecan in first-line setting. Bevacizumab was added later when it became standard in first-line metastatic CRC patients. Fifty-seven patients received bevacizumab with infusional 5FU, leucovorin and irinotecan (FOLFIRI) and 60 received modified IFL (mIFL) with bevacizumab. The 1-year survival was 87 and 61% in the FOLFIRI plus bevacizumab and mIFL plus bevacizumab groups, respectively. In contrast, the 1-year survival in the FOLFIRI and mIFL groups was 75 and 65% respectively. Readers should be aware that the survival between bevacizumab-containing and bevacizumab-noncontaining groups cannot be compared directly since this was not the primary objective of the study.

The role of bevacizumab in the second-line setting in CRC was examined in the Eastern Cooperative Group Study E3200 *(10)*. Eight hundred and twenty-nine patients with metastatic disease previously treated with irinotecan and fluoropyrimidine were randomly assigned to

receive oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) with bevacizumab, FOLFOX4 alone, and bevacizumab alone. Compared to FOLFOX4-alone arm, the addition of bevacizumab to FOLFOX4 achieved superior median survival (12.9 vs. 10.8 months; hazard ratio [HR] 0.75, $p = 0.0011$), progression-free survival (7.3 vs. 4.7 months, HR 0.61, $p < 0.0001$) and response rate (22.7 vs. 8.6%, $p < 0.0001$). Hypertension and bleeding were more frequent among the patients receiving bevacizumab.

Bevacizumab seemed to enhance the efficacy of oxaliplatin-containing regimens in the first-line setting in two sequential trials, TREE-1 and TREE-2 *(11)*. In the TREE-1 trial, patients with untreated metastatic CRC were randomized to receive three oxaliplatin-containing regimens based on infusional 5FU regimen (FOLFOX), bolus 5FU regimen (bFOL), and capecitabine regimen (CAPOX). Bevacizumab was later added to the above regimens (TREE-2). When all arms were compared, the bevacizumab-containing regimens had higher response rate and better time-to-progression than the regimens without bevacizumab. It is important to note that the two trials were conducted sequentially and like the BICC-C trial, the bevacizumab-containing and bevacizumab-noncontaining arms were not compared in a head-to-head fashion.

The role of maintenance bevacizumab, with or without erlotinib, is being explored in the DREAM trial (Double Reintroduction with Erlotinib and Avastin in Metastatic Colorectal Cancer) *(12)*. The study plans to enroll 640 patients with newly diagnosed metastatic CRC to be treated with bevacizumab plus FOLFOX or CAPOX4 (capecitabine plus oxaliplatin) for six cycles. The patients will then be randomized to receive bevacizumab alone or bevacizumab plus erlotinib after completion of the six cycles or during chemotherapy-free period. The cytotoxic regimens will be reintroduced during disease progression.

2.2. Sunitinib

Sunitinib is an oral inhibitor of VEGFR-2, PDGFR, c-kit, and FLT-3, and has preclinical anti-tumor activity in a number of cancer models *(13–15)*. The toxicities include hypertension, thrombocytopenia, neutropenia, diarrhea, and hair and skin changes *(16)*. Similar to bevacizumab, sunitinib did not have single-agent activity in treatment-refractory metastatic CRC in clinical trial *(17)*. Sunitinib was found to be tolerable at 37.5 mg/day on a "4-weeks-on/2-weeks-off" schedule when combined with irinotecan-containing regimen (FOLFIRI) in a dose-finding study *(18)*. The investigators are exploring a continuous schedule of sunitinib at 37.5 mg/day in combination with FOLFIRI.

3. EPIDERMAL GROWTH FACTOR RECEPTOR PATHWAY

The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase, belonging to the family of human epidermal growth factor receptor (HER)-kinases which includes EGFR (Erbb1), HER-2 (Erbb2), Erbb3, and Erbb4 *(19)*. Upon ligand binding, EGFR pairs with another EGFR molecule (homodimerization) or other members of the HER family receptor kinases (heterodimerization). The activating ligands of EGFR include epidermal growth factor (EGF) and transforming growth factor (TGF-α). This leads to receptor autophosphorylation and activation of intracellular signaling pathways, such as mitogen-activated protein kinase (MAPK) and Akt/mTOR (mammalian target of rapamycin) axes, which regulate cellular proliferation, differentiation, adhesion, survival, and migration *(20)*. Overexpression and upregulation of EGFR were found in CRCs and were associated with early progression and poor survival *(21–23)*. These led to interests in exploiting this pathway in anticancer therapy as either single agent or in combination therapies *(24)*. Targeting the EGFR with monoclonal antibodies has been the most successful strategy in the clinic so far.

3.1. Cetuximab

Cetuximab is a chimeric murine–human IgG1 monoclonal antibody with high specificity and affinity for EGFR. The antibody blocks ligand-dependant autophosphorylation of EGFR. The agent demonstrated primarily cytostatic properties when used as single agent in preclinical models, but was synergistic with irinotecan in irinotecan-refractory CRC xenograft models *(25–27)*. Cetuximab was the first in its class to be approved by the US FDA for use in cancer patients.

Cetuximab was found to be active in metastatic colorectal patients who failed previous irinotecan-containing therapies in two phase II studies *(28, 29)*. The response rate with cetuximab single-agent therapy was 9 and 17% when administered with irinotecan. In a separate multi-institutional randomized study (the BOND study), 329 metastatic CRC patients who failed previous irinotecan-containing regimens were randomized to receive either cetuximab plus irinotecan or cetuximab monotherapy *(30)*. The patients who received the combination of cetuximab and irinotecan achieved superior time to progression (4.1 vs. 1.5 months; $p < 0.001$) and response rate (22.9 vs. 10.8%; $p < 0.01$) than the cetuximab monotherapy group. However, median survival times were not significantly different between both groups (8.6 vs. 6.9 months; $p = 0.48$).

Preclinical models suggested correlation between tumor EGFR positivity and cetuximab efficacy, leading to selection of EGFR-expressing metastatic CRC patients in the above trials. As such, cetuximab therapy was approved by US FDA for use in patients with EGFR-expressing CRC. However, later studies suggested that the degree of EGFR expression determined by contemporary immunohistochemical technique, did not affect response to cetuximab *(31, 32)*. It is generally recommended that patients with EGFR-negative CRC should not be deprived of this agent when indicated.

The role of cetuximab in first-line CRC therapy is being investigated. In a single-arm phase II study, 43 patients with previously untreated metastatic CRC received cetuximab with an oxaliplatin-containing regimen (FOLFOX4) and achieved a response rate of 72% and a median survival of 30.0 months *(33)*. In a multicenter randomized study (the OPUS trial), patients with previously untreated EGFR-expressing metastatic CRC were randomized to receive FOLFOX4 with or without cetuximab *(34)*. Preliminary analysis of 337 patients showed significantly higher response rate in the cetuximab-containing arm, while results on progression-free survival and overall survival were pending. Rash was more frequent in the cetuximab-containing group; otherwise, there was no significant difference in toxicities between both arms. Other large randomized trials examining the benefit of cetuximab with infusional 5FU-based regimens (FOLFOX or FOLFIRI) in first-line setting, such as the EXPLORE and CALGB 80203 trials, were plagued by poor patient accrual *(35, 36)*.

3.2. Panitumumab

Panitumumab is a fully humanized monoclonal antibody against EGFR that was approved by the US FDA for treatment of patients with EGFR-expressing metastatic CRC refractory to oxaliplatin-, irinotecan-, and fluoropyrimidine-containing chemotherapy regimens *(37)*. In the phase III open-labeled trial, 463 patients with EGFR-expressing metastatic CRC were randomized to receive panitumumab and best supportive care (BSC) or BSC alone *(38)*. The group receiving panitumumab achieved significantly better progression free survival (HR 0.54, 95% CI 0.44–0.66) than those receiving BSC alone. The response rate was 10% for the panitumumab group compared to 0% in the BSC-alone group. Common toxicities included skin rash, hypomagnesaemia and diarrhea, which were manageable.

In view of the encouraging single-agent activity, panitumumab was developed further in the first line setting in metastatic CRC patients. The Panitumumab Advanced Colorectal Cancer Evaluation (PACCE) study was a large phase III open-labeled study that enrolled previously

untreated metastatic CRC patients to receive oxaliplatin- or irinotecan-based chemotherapy plus bevacizumab, with or without panitumumab. The study was discontinued when preplanned interim analysis revealed that the overall survival of patients receiving panitumumab was inferior to the control arm (HR 1.44; 95% CI 1.10–1.88) *(39, 40)*. There was also an increased incidence of serious adverse events in the panitumumab-containing arm.

3.3. Genetic Selection in Colorectal Cancer: KRAS Mutations and Efficacy of Anti-EGFR Therapy

After a series of non-randomized studies reporting little or no benefit from anti-EGFR therapies as single agents or combined with chemotherapy in subjects with *KRAS* mutant tumors, *(41–46)* evidence from randomized studies has become available (Tables 3 and 4). In summary, CRC patients harboring a *KRAS* mutation do not derive benefit from the administration of EGFR-targeting monoclonal antibodies in the first-line, *(47, 48)* second-line, *(49)* or third-line settings *(50)*. The compelling nature of this retrospective data obviates a need for prospective data collection before action was taken, ad the regulatory bodies in Western countries have adapted their approved profiles for both cetuximab and panitumumab limiting their use in patients with *KRAS* wild-type CRC.

Table 3
Results from the EGFR Inhibitor-Containing, Single-Arm CRC Studies Analyzing the Correlation of Efficacy and *KRAS* Status

			Response rate (%)	
Study	*Treatment*	*Total Pts*	KRAS *MT*	KRAS *WT*
Benvenuti 2007[3]	P or C or C ± CT	48	6	31
De Roock 2007[4]	C ± CT	113	0	40
Di Fiore 2007[6]	C + CT	59	0	28
Khambata 2007[7]	C	80	0	10
Lievre 2008[8]	C ± CT	89	0	40

C, cetuximab; CRC, colorectal cancer; CT, chemotherapy; MT, mutant; P, panitumumab; WT, wild-type.

Table 4
Results from the EGFR inhibitor-containing arms from CRC randomized studies analyzing the correlation of efficacy and *KRAS* status

Study	*Treatment*	*Total Pts*	KRAS *MT PFS*	KRAS *WT PFS*
Amado 2008[12]	P versus BSC (3rd line)	427	7.4 weeks HR 0.99	12.3 weeks HR 0.45
Van Cutsem 2008[9]	FOLFIRI ± C (1st line)	540	7.6 months HR 1.07	9.9 months HR 0.68
Bokemeyer 2008[10]	FOLFOX ± C (1st line)	233	5.5 months HR 1.83	7.7 months HR 0.57

BSC, best supportive care, C, cetuximab; CRC, colorectal cancer;FOLFIRI, bolus & infusional 5FU + leucovorin + irinotecan; FOLFOX, bolus & infusional 5FU + leucovorin + oxaliplatin; HR, hazard ration; MT, mutant; P, panitumumab; PFS, frogression-free survival; WT, wild-type.

4. COMBINED TARGETING OF ANGIOGENESIS AND EGFR

Dual targeting of angiogenesis and EGFR pathway in CRC seems rational following the success of individual targeting of angiogenesis and EGFR pathway. The combination of erlotinib (a small-molecule inhibitor of EGFR tyrosine kinase) with FOLFOX and bevacizumab proved to have unacceptable toxicities in a phase II study *(51)*. Thirty-five patients with previously untreated metastatic CRC were treated with the combination and all patients were taken off study for reasons other than disease progression. About 75% of the patients discontinued treatment due to toxicities, indicating that this combination at the prescribed dosing schedule was not feasible clinically.

In contrast, the approach of combining anti-EGFR monoclonal antibody, cetuximab, with bevacizumab and FOLFOX6 proved to be tolerable *(52)*. This phase II trial aimed to test the combination of cetuximab, bevacizumab, and FOLFOX6 in metastatic CRC patients in first-line setting. Preliminary analysis of 67 patients reported a response rate of 55%, median progression-free survival of 9.6 months and 71% remained progression free for at least 8 months. The toxicities were reportedly tolerable and included neutropenia, thromboembolic disease, and rash.

The BOND-2 trial examined the efficacy of combining cetuximab and bevacizumab with irinotecan in irinotecan-refractory metastatic colorectal patients *(53)*. Patients were randomly assigned to receive cetuximab and irinotecan with or without bevacizumab. The bevacizumab-containing arm achieved a higher response rate (37 vs. 20%) and median survival (14.5 vs. 11.4 months) when compared to the arm without bevacizumab. The toxicities were manageable and were not higher than expected. However, the trial was closed early due to poor accrual, thus limiting the interpretation of the efficacy of the combinations *(40)*.

The CALGB 80405 trial is a multi-institutional randomized controlled trial currently underway and will hopefully definitively clarify the role of dual targeting of EGFR and angiogenesis, with cetuximab and bevacizumab, in combination with contemporary colorectal regimens, FOLFIRI or FOLFOX, in first-line setting *(54)*.

5. CONCLUSION

The survival of metastatic CRC patients has improved significantly over the last few decades and was possible with the advent of several active antineoplastic agents. Conventional cytotoxics that interrupts tumor DNA synthesis and repair mechanisms dominated the early development of CRC therapy. A new class of agents targeting biological processes in tumors was synergistic with these cytotoxics, and angiogenesis and EGFR pathway proved to be valid targets in CRC therapy.

Bevacizumab is the first antiangiogenic agent to demonstrate efficacy in CRC patients. This monoclonal antibody is active in first and second-line settings when combined with fluoropyrimidine-based regimens. The role of bevacizumab-based maintenance therapy is currently under investigation. EGFR pathway seems to be best exploited by a monoclonal antibody-based approach. Cetuximab had demonstrated clinical activity in irinotecan-refractory patients and its role in the first-line setting remains to be defined. Perhaps the most anticipated question is whether simultaneous targeting of both pathways will yield superior clinical outcome, or whether a sequential exposure to these agents is the more optimal approach. Panitumumab's success in treatment-refractory CRC patients is opposed to its failure in first-line setting when combined with cytotoxics is puzzling; understanding of the underlying reason will benefit future therapy development.

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44 Developments in the Management of Genitourinary Malignancies: Prostate Cancer and Renal Cell Carcinoma

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CONTENTS

ABSTRACT

Recent years have brought to the clinic a series of novel treatment approaches in the urologic malignancies, in particular, in patients with renal cell carcinoma and prostate cancer. Selected novel therapies will be highlighted.

KeyWords: castrate-resistant prostate cancer; adrenal androgens; immunotherapy; phase II; abiraterone; atrasentan; bevacizumab; calcitriol; chemotherapy; GVAX; ipilimumab; ixabepilone; satraplatin; sipuleucel-T; VEGF; sunitinib; sorafenib

1. PROSTATE CANCER

Although advanced prostate cancer is amenable to control through androgen deprivation, once this modality ceases to be effective there are relatively few treatment options. Recently, several new approaches (Table 1) have shown promise including strategies that target the androgen: androgen receptor interaction, chemotherapy, and immunotherapeutics.

1.1. Secondary Hormonal Manipulations

A series of recent studies suggest that intratumor androgens coupled with continued activation of the androgen receptor may mediate progression of this disease to what is termed castration-resistant prostate cancer (CRPC) *(1)*. "Adrenal" androgens such as androstenedione and dihydroepiandosterone sulfate (DHEAS) are also thought to activate the androgen receptor in this setting. Thus, the development of therapies capable of interrupting this interaction hold

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Table 1
Novel Agents in Development for Prostate Cancer

Drug	*Presumed mechanism*
	Secondary hormonal therapy
Abiraterone	Adrenal androgen inhibitor
MDV 3100	Androgen receptor antagonist
	Cytotoxic chemotherapy
Ixabepilone	Epothilone B analog—microtubule-stabilizing agent
	Endothelin antagonist
Atrasentan	Endothelin A receptor antagonist
	Anti-VEGF
Bevacizumab (with Docetaxel)	Monoclonal antibody that targets VEGF
Sorafenib	Tyrosine kinase inhibitor
	Immunotherapy
Sipuleucel-T	Antigen-presenting cells pulsed with PAP fused to GM-CSF
GVAX	Cellular vaccine, used exogenous tumor cells engineerd to secrete GM-CSF
Ipilimumab	Anti-CTLA-4 antibody

promise in the area of CRPC. Abiraterone acetate (CB7630—Cougar Biotechnology, Los Angeles, CA) is an orally available inhibitor of 17-alpha hydroxylase and C17, 20-lyase, both of which result in the production of adrenal androgens. Phase I and II data with this agent suggest a high level of activity in CRPC. Preliminary results from one such early study have been reported. In this study of 34 patients, a 50% prostate-specific antigen (PSA) decline was observed in 21/34 (60%) with a PSA decline of >90% was observed in 11/34 (32%) of patients, and objective (radiologic) partial responses were seen in 12/21 (57%) of patients with disease evaluable by RECIST (Response Evaluation Criteria in Solid Tumors) *(2)*. Preliminary results from a second study involving 16 patients, (three with nonmetastatic CRPC and 13 with bone/soft tissues metastases), have also been reported *(3)*. Of the 14 patients receiving at least one month of abiraterone, a 50% PSA decline was seen in seven (50%) patients. Benefit has been observed even in patients who had undergone prior treatment with ketoconazole, another adrenal androgen inhibitor. Of the nine patients who were refractory to ketoconazole, five experienced >50% PSA declines on abiraterone. Although changes in PSA have not been fully validated as a surrogate endpoint, they are commonly used *(4,5)*. Additional studies of abiraterone in patients with metastatic disease both prior to and after chemotherapy are underway.

Consistent with a mechanism of action that leads to preferential inhibition of the formation of androgens by the adrenal gland with a relative sparing of the glucocorticoid and mineralocorticoid precursors, increased elevels of upstream steroid precursor levels, including an increase in corticosterone have been observed as adrenal androgen levels also decreased. The increase in mineralocorticoid precursors has been accompanied by toxicities consisting of hypertension and hypokalemia. Clinically significant adrenal insufficiency has not been consistently observed to date. This agent will enter definitive phase III testing in 2008 in men with CRPC who have received prior docetaxel chemotherapy.

1.2. MDV3100

MDV3100 (Medivation, Inc., San Francisco, CA) is a small-molecule antagonist of the androgen receptor. Development of this compound arose from high throughput screening of compounds based on profiling studies demonstrating that an increase in androgen receptor mRNA was a consistent change associated with the development of resistance to antiandrogen therapy *(6)*. The resultant compound (MDV3100) maintained antagonistic properties in the setting in which standard antiandrogens functioned as agonists. Preclinical data showing a more potent inhibition of this agent than the most commonly utilized antiandrogen, Casodex, have prompted phase I open-label, uncontrolled, dose-escalation study of MDV3100 from which there is preliminary evidence of clinical activity. Further study of this agent is ongoing.

1.3. Chemotherapy for Castration-Resistant Prostate Cancer

More than 3 years have elapsed since the US Food and Drug Administration (FDA) approved the use of docetaxel for metastatic hormone-refractory prostate cancer (HRPC). This approval resulted from two simultaneous phase III randomized trials conducted to demonstrate improved survival in patients treated with first-line docetaxel as compared to mitoxantrone, the previous standard chemotherapy (a therapy which had not demonstrated survival improvements over prednisone). Study 9916 from the Southwest Oncology Group (SWOG 9916), compared docetaxel plus estramustine phosphate to M/P (Mitoxantrone was administered at a dose of 12 mg/m^2 q 21 days and prednisone was 5 mg po bid). The multinational study Tax 327 utilized three treatment arms: docetaxel (35 mg/m2) administered weekly versus docetaxel (75 mg/m^2) given every 3 weeks versus mitoxantrone 12 mg/m^2, which is also administered every 21 days. In the SWOG study, 88% had bony metastases and 36% had pain requiring opiate analgesics. Likewise, in Tax 327, all patients had metastatic HRPC, 90% had metastatic disease in the bone and approximately one third had disease related pain requiring opiate analgesic use.

The results of the two studies demonstrated convergent results. In SWOG 9916, the median survival in the docetaxel arm was 18.9 months compared to 16 months for those patients treated with mitoxantrone, associated with a hazard ratio (HR) for death of 0.80 (95% CI 0.67, 0.97) $p = 0.01$ when compared to M/P. In the Tax 327 study, docetaxel q 3-week arm was 18.9 months, which was significant when compared to the 16.4 months in those treated with M/P and translated into a HR of 0.76 and a p value of 0.009. Survival of patients treated on the weekly docetaxel arm was 17.3 months, not significantly different than patients treated with M/P. Docetaxel was also superior to Mitoxantrone with respect to pain response rate (35 vs. 22%; $p = 0.01$) and proportion of patients who experienced a >50% decline in PSA (45 vs. 32%; $p = 0.0005$). Based on these findings, the US FDA approved the use of docetaxel (75 mg/m^2 every 21 days) together with prednisone as frontline therapy for metastatic HRPC in May 2004 *(7,8)*.

While these results have led to the establishment of docetaxel as the standard of care for HRPC, several other chemotherapy approaches to improving its efficacy are under development.

1.4. Epothilone Analogs: Ixabepilone

Ixabepilone (BMS-247550) is a synthetic epothilone B analog utilized as a microtubule-stabilizing agent. In vitro, ixabepilone demonstrates activity in taxane-resistant prostate cancer. Phase II evaluations of single agent ixabepilone demonstrated a 33% PSA response proportion 42 chemotherapy-naïve patients *(9)*. Data from a randomized phase II study of ixabepilone or mitoxantrone/prednisone in patients with docetaxel-refractory CRPC have been reported *(10)*. In this study, 17% of the 41 patients had at least a 50% decline in PSA. A phase I/II trial of ixabepilone in combination with mitoxantrone and prednisone as second-line chemotherapy for metastatic CRPC is underway.

1.5. Chemotherapy Plus Angiogenesis Inhibition

One attractive approach is the addition of an angiogenesis inhibitor to docetaxel-based therapy, as has proven efficacious in other solid tumors.

Vascular endothelial growth factor (VEGF), an angiogenesis factor, is increased in men with metastatic prostate cancer and is associated with a poor prognosis *(11,12)*. Bevacizumab, is a humanized monoclonal antibody that targets VEGF. Monotherapy with bevacizumab did not show efficacy in patients with metastatic CRPC, although it has the potential to be efficacious when given in combination with chemotherapy *(13)*. While the mechanism of this benefit is incompletely understood, it likely involves a "normalization" of blood vessels in the tumor with resulting decreased vascular permeability, resulting in increased chemotherapy delivery to the tumor itself *(14)*. Based on this hypothesis and supporting phase II data of the combination of docetaxel and bevacizumab improves chemotherapy delivery, a phase III trial comparing docetaxel and prednisone with or without bevacizumab has been conducted by Cancer and Leukemia Group B (CALGB) in the USA. This trial is fully accrued with over 1,000 patients, giving it the statistical power to detect a 20% improvement in survival with the addition of bevacizumab. Data are expected in 2009 or 2010.

Thalidomide, another agent capable of mitigating the effects of VEGF in tumors, has been given in chemotherapy combination strategies *(15,16)*. In one phase II study, 39 patients were treated with combinations of thalidomide, bevacizumab, and docetaxel. Thirty-four patients (87%) had PSA declines of $\geq$50%, with a median response duration of 12 cycles. Seventeen patients with measurable disease were evaluable; of these, one patient had a complete response and nine had partial responses, for a 59% overall response rate. Although further work with these agents is clearly required, these results suggest that the combination of standard chemotherapy with antiangiogenic agents is promising.

1.6. VEGF Tyrosine Kinase Inhibition

Oral agents that target the VEGF receptor tyrosine kinase have been studied in CRPC. Sorafenib is a small molecula that inhibits b- and c-Raf kinase, platelet-derived growth factor receptor (PDGFR), c-kit, VEGFR, and Flt-3 *(17)*. Phase II trials have evaluated the use of sorafenib in CRPC. In one study 22 patients with metastatic CRPC were given continuously dosed sorafenib in an open-label, single arm study *(18)*. Interestingly, two patients had dramatic improvements on bone scan although they met PSA progression criteria, suggesting PSA may not be an appropriate marker for monitoring sorafenib activity. In a second study, 55 patients with chemotherapy-naïve CRPC received continuously dosed sorafenib *(19)*. Of forty-two evaluable patients; two patients (4.8%) had a response and 15 patients (35.7%) demonstrated stable disease for $\geq$12 weeks. While these results suggest that sorafenib is capable of inducing rare responses and stable disease in many, toxicity with this agent can be significant and substantial efficacy may need to be demonstrated to make sorafenib a useful therapeutic in prostate cancer.

1.7. Other Investigational Agents

1.7.1. Atrasentan

Endothelin is a protein produced by vascular endothelium and thought to play an important role in vascular homeostasis. Endothelin-1 secretion is common in metastatic prostate cancer, and it is hypothesized that the binding of endothelin-1 to endothelin receptors on osteoblasts may promote progression of prostate cancer in bone *(20)*. Atrasentan is a selective endothelin A

(ET_A) receptor antagonist (Abbott Laboratories, Abbott Park, IL). A phase III trial randomized 941 men with nonmetastatic CRPC and serologic (PSA) progression to atrasentan or placebo *(21,22)*. While there were no statistically significant difference observed with respect to time to disease progression was observed, a trend toward improved survival was observed in the atrasentan arm with a median survival of 121.2 months for the atrasentan arm and 115.2 months for the control arm (HR = 0.909, $p = 0.176$). Based on this observation, a second phase III trial comparing docetaxel/prednisone and atrasentan to docetaxel/prednisone and placebo in 706 men with metastatic CRPC was initiated (SWOG S0421) and results are pending. Adverse effects common during therapy with this agent include peripheral edema, rhinitis, dyspnea, and headache.

1.8. Immunotherapy

A significant body of preclinical data suggest that prostate cancer may be amenable to immunotherapy *(23)*. Multiple immune-based treatment strategies are in development and are described below.

1.8.1. Sipuleucel-T

Dendritic cells, the most potent and prevalent antigen-presenting cells in normal physiology, are known to be deficient in number and functional activity in the setting of cancer *(24,25)*. Sipuleucel-T (Dendreon, Inc., Seattle, WA) is a treatment based on the technology of presentation of antigen under ex vivo conditions in an attempt to stimulate a T cell immune response. To produce sipuleucel-T, human prostatic acid phosphatase (PAP) is fused to a granulocyte-macrophage colony-stimulating factor (GM-CSF) cassette, which targets the protein to cells expressing the GM-CSF receptor. Patients undergo leukapheresis three times to remove unstimulated dendritic cells, which are reinfused after 40 h of ex vivo stimulation.

The results of a randomized, placebo-controlled phase III study of sipuleucel-T in 127 men with metastatic has generated considerable enthusiasm for this approach *(26)*. The primary endpoint was time to objective disease progression, which did not include PSA progression. The median time to progression (TTP) for sipuleucel-T was 11.7 weeks, compared with 10.0 weeks for placebo ($p = 0.052$). although survival was not a primary endpoint of the study, a difference in survival was observed in favor of those patients who received vaccination. Three-year overall survival was 34% in the sipuleucel-T group versus 11% in the placebo group ($p = 0.0046$). This treatment is generally well-tolerated although reinfusion is associated with rigors, pyrexia and tremors in many patients. A review by the US FDA of the above data resulted in the granting of "approvable" status to this approach, thus requiring further phase III data prior to full approval. Phase III studies are underway.

1.8.2. Cellular Vaccination

Another vaccine approach is to treat patients with live, attenuated prostate cancer cells. GVAX (Cell Genesys, South San Francisco, CA) is a cellular vaccine that uses exogenous tumor cells engineered to secrete GM-CSF to increase dendritic cell presentation of antigens to the immune system *(27)*. Two cell lines, PC-3 and LNCaP, comprise the GVAX vaccine. The multitude of antigens contained in these cell lines, in concert with the high concentration of GM-CSF, induces dendritic cell antigen presentation and activation of effector cells, such as T cells and macrophages *(28)*.

Phase II studies of GVAX have included 114 patients. Lacking a valid surrogate marker of immune activation against prostate cancer, the results of these studies are presented as actual

survival compared to that predicted by a pretreatment prediction nomogram *(29)*. In one phase II study, the median survival was 26.2 months, compared to the 19.5 months ($p = 0.01$) predicted by the pretreatment nomogram *(30)*. In another study utilizing a form of the vaccine was reengineered to secrete a higher dose of GM-CSF, the median survival was 35.0 months *(31–33)*. Based on these findings, two phase III studies evaluating GVAX to docetaxel-based chemotherapy were initiated. One study (VITAL-1), compares GVAX directly to docetaxel plus prednisone. In the second (VITAL-2), treatment with GVAX plus docetaxel (without prednisone) is compared to docetaxel plus prednisone alone. Vital 1 has finished accrual and accrual to Vital 2 is ongoing.

1.8.3. Anti-CTLA4 Therapy: Ipilimumab

Immunotherapy is likely to depend on a high degree of activation of T cells. Such activation requires that a T cell receptor recognize specific antigenic peptides in the context of major histocompatibility complex (MHC) molecules expressed dendritic cells (DC). Costimulatory molecules are required to both enhance and attenuate this process. Ligand engagement by CD28 stimulates T cells and interactions between CTLA-4 and these ligands inhibit T cell stimulation. Prevention of the interaction of CTLA-4 and its ligands using an antibody is thus hypothesized to augment immune responses against antigens that would not generally stimulate a robust immune response. Thus, blockade of CTLA-4 may potentiate T cell stimulation, and is sought as a treatment strategy.

Ipilimumab (Medarex, Princeton, NJ) is a humanized anti-CTLA-4 antibody, under evaluation in prostate cancer and other malignancies. In phase I studies of anti-CTLA-4 antibody in patients with prostate cancer, 14 patients were given one dose of humanized anti-CTLA-4 antibody. Two patients demonstrated PSA declines of at least 50% and two patients demonstrated prolongation of their PSA doubling times (PSADT) *(34)*. A phase I trial combining ipilimumab with GM-CSF, as a means of enhancing antigen presentation, is currently underway *(35)*. In this study, treatment with ipilimumab at 3 mg/kg plus GM-CSF resulted in >50% PSA declines in three of six patients. Two of the three patients who had PSA declines also experienced toxicity in the form of autoimmunity, with National Cancer Institute (NCI) Common Toxicity Criteria grade III panhypopituitarism and rash in one, and grade III colitis in another. Autoimmunity appear to correlate with responses in other malignancies that have been treated with ipilkmumab *(36)*.

1.9. Prostate Cancer: Conclusions

While the current standard of care for metastatic CRPC is a combination of docetaxel every 3 weeks and prednisone, additional treatment approaches for patients with castrate-resistant disease are under development. Accrual to studies of these diverse agents is required to move the field forward.

2. RENAL CELL CARCINOMA

Among the urologic malignancies, medical progress has been greatest in the area of kidney cancer. Until recently, the treatment options for advanced kidney cancer had been extremely limited. Cytotoxic chemotherapy is regarded as ineffective against this disease and immunotherapy with interleukin (IL)-2 or interferon had previously been the standard treatment despite only marginal efficacy. High-dose IL-2 approved by the FDA for the treatment of advanced kidney cancer on the basis of a 3–5% durable complete response rate, despite no proof of a prolongation of median survival in randomized clinical trials. Further, the substantial toxicity of high-dose IL-2 limited its use. Low-dose interferon is associated with low objective response rates, infrequent durable responses, and similarly significant side effects. These relatively ineffective approaches have prompted the search for more effective systemic therapies.

2.1. Drug Targets in Renal Cell Carcinoma

2.1.1. VHL in Kidney Cancer

It has long been recognized that clear cell renal cell carcinoma (CCRCC—the most common subtype) is a highly vascularized tumor. The relationship between vascularity and disease pathogenesis in CCRCC became apparent with the identification the von Hippel–Lindau (VHL) tumor suppressor gene located on chromosome 3p25-26 in the 1990s. Also, an association between VHL syndrome and clear cell kidney cancer has long been recognized. VHL mutation or loss is evident in approximately 90% of all CCRCCs *(37)*. Sporadic tumors are also found to have high frequencies of VHL loss of heterozygosity, mutation, and methylation (that silences VHL gene transcription).The elucidation of VHL's central role in the pathogenesis of clear cell kidney cancer has led to the development of targeted agents with clinical benefits in the treatment of advanced kidney cancer *(38)*.

Of its many functions, the VHL protein forms a complex with hypoxia-inducible factor (Hif). Hif1α and Hif2α act as transcription factors to promote transcription of hypoxia-related genes. The VHL complex tags the Hifs for ubiquitin-mediated degradation *(39)*. Thus, mutation, deletion, or methylation of the VHL gene lead to decreased VHL protein function in turn leading to accumulation of Hif1α resulting in a net increase in the mediators of neovascularization and angiogenesis, principally VEGF. VHL-driven elevated VEGF expression is frequent in clear cell kidney cancer, and is responsible for the highly vascularized nature of these tumors. Both VEGF and PDGF are targets of Hif1α transcription and mediate neovascularization as well as maintenance of existing blood vessels. VEGF and PDGF tyrosine kinase receptors are found on endothelial cells and pericytes and are responsible for mediating migration, growth, and proliferation signals to the microvasculature. Activation of the VEGF and PDGF receptors on endothelial cells lead to downstream cascades of events resulting in tumor angiogenesis.

2.1.2. mTOR in Kidney Cancer

The mammalian target of rapamycin (mTOR) protein also plays a role in Hif1α accumulation and VEGF overexpression in RCC. mTOR, a serine–threonine kinase, is a downstream effector of critical cell regulatory pathways and plays a critical role in cell growth and proliferation. In preclinical models, mTOR expression increases tumor cell proliferation and angiogenesis. Althought mutation of mTOR is not seen in cancer, upregulation and activation of pathways that signal growth and proliferation are mediated via mTOR activity.

mTOR inhibition results in decreased tumor growth and angiogenesis. mTOR inhibitors bind to FKBP12, which then binds to mTOR. FKBP binding inhibits the kinase activity of mTOR, leading to decreased downstream signaling through this pathway. Because mTOR activation results in increased translation and accumulation of Hif1α and Hif2α, there is potential synergy with VHL/VEGF-targeted approaches *(40)*. In addition, mTOR activation is observed in familial RCC associated with the tuberous sclerosis syndrome, suggesting a pathologic link between the mTOR and the development/progression of kidney cancer *(41)*.

2.2. VEGF Pathway-Directed Angiogenesis Inhibitors

2.2.1. Bevacizumab

The first studies targeting VEGF in RCC utilized bevacizumab, a humanized monoclonal antibody that binds circulating VEGF-A. Yang et al. at the National Cancer Institute conducted a double-blind, randomized phase II study of two doses of bevacizumab or placebo in previously treated patients with advanced kidney cancer *(42)*. Objective responses rates in this study were

low (10% in the high-dose arm); however, those who received high-dose bevacizumab demonstrated a significantly longer TTP compared to placebo (4.8 vs. 2.5 months). An survival advantage in bevacizumab-treated patients was not observed, potentially because the study allowed patients initially treated with placebo to cross over and receive high-dose bevacizumab. Although the improvements associated with bevacizumab were modest, they provided a signal that anti-VEGF-directed therapy held promise.

Two large phase III studies evaluating interferon with or without bevacizumab have completed accrual, and the results are pending at this time. In the European AVOREN study, 649 patients were randomized to therapy with interferon versus interferon plus bevacizumab. Results demonstrated that the addition of bevacizumab to interferon therapy significantly increased progression-free survival (10.2 vs. 5.4 months) (HR = 0.63; $p < 0.0001$) and objective tumor response rate (30.6 vs. 12.4%; $p < 0.0001$) *(43)*. Overall survival was not improved to a statistically significant degree with the addition of bevacizumab ($p = 0.0670$), perhaps reflecting the fact that many patients in the interferon-alone arm received VEGF-targeted therapy at the time of clinical progression. Results from a similar north American study of this combination are pending.

2.2.2. Sunitinib

Sunitinib is a small-molecule tyrosine kinase inhibitor of the VEGF and PDGF receptor as well as the c-kit oncogene. The drug is administered at a dose of 50 mg daily for 28 days followed by a 14-day break, in repeated cycles. Initial clinical testing of this agent was performed in patients with cytokine (e.g., interferon or IL-2)-refractory kidney cancer. Partial responses were observed in 34–40% of patients treated, and a median TTP of 8.3–8.7 months. *(44*, Motzer, 2006, *45)*, results which compared favorably to outcomes of historical series of cytokine refractory patients.

Based on these results, a randomized phase III study of sunitinib versus interferon-α was conducted in 750 untreated patients with metastatic RCC *(46)*. Sunitinib-treated patients experienced a progression-free survival of 11 months, compared to 5 months for patients receiving interferon-α ($p < 0.001$). Objective responses occurred in 31% of sunitinib-treated patients, compared to 6% of interferon-treated patients ($p < 0.001$). Nearly all of the observed responses were partial responses. One patient (out of 374) treated with sunitinib experienced a complete response. Toxicities associated with sunitinib included diarrhea, fatigue, nausea, vomiting, hypertension, hand–foot syndrome, and decreased ejection fraction (in approximately 4%) plus hypothyroidism. Despite the increased side effects seen with sunitinib, patients receiving sunitinib reported an improved quality of life compared with patients receiving interferon-α *(47)*. Sunitinib was approved by the FDA in 2006.

2.2.3. Sorafenib

Sorafenib is a small-molecule inhibitor of multiple receptor tyrosine kinases, including VEGF receptors 1, 2, and 3, PDGFRβ, raf kinase, RET receptor kinase, and c-kit. In animal models, sorafenib demonstrated substantial inhibition of kidney cancer growth and angiogenesis *(48)*.

Sorafenib was studied in CCRCC utilizing a novel trial design called a phase II *randomized discontinuation* study *(49)*. Patients receiving sorafenib whose best response was stable disease at 12 weeks were randomized to either continue sorafenib or receive a placebo. Patients continuing sorafenib experienced an improved progression-free survival compared to placebo (24 vs. 6 weeks, $p = 0.0087$). These results formed the basis of a randomized phase III study of sorafenib compared with placebo in patients with previously treated kidney cancer. In that study, 903

CCRCC patients with progressive disease despite prior therapy were randomized to either sorafenib or placebo *(50)*. Patients receiving sorafenib experienced a 5.5-month median progression-free survival, compared with 2.8 months in the placebo group ($p < 0.01$). Partial responses were observed in 10% of patients receiving sorafenib and 2% of patients receiving placebo. Like sunitinib, sorafenib is associated with multiple toxicities, including diarrhea, rash, fatigue, and hand–foot skin reactions. In this study those receiving placebo were allowed to receive sorafenib at the time of disease progression, and overall survival data from this trial continue to mature at this time. The effects of crossover may blunt the ability to determine an overall survival advantage for sorafenib, although the data are still maturing. Sorafenib is now FDA approved and commercially available. Treatment is given at a dose of 400 mg orally twice daily, given without treatment breaks.

Other VEGF receptor-targeted agents are in development in advanced kidney cancer. Axitinib is an agent associated with a 46% PR rate and a 40% prolonged stable disease rate *(51)*. Pazopanib has likewise been studied in a randomized discontinuation design and is associated with a high degree of activity.

2.3. mTOR Inhibition

2.3.1. Temsirolimus (CCI-779)

Based on the observations described above, clinical mTOR inhibition is an active area of investigation in advanced kidney cancer. Temsirolimus is an agent administered intravenously on a weekly basis that has recently been approved for use in advanced high-risk kidney cancer *(52)*. After a phase I dose escalation trial of temsirolimus failed to demonstrate a pharmacologic maximum tolerated dose, a dose ranging randomized phase II study was conducted *(53)*. A retrospective analysis revealed that poor-risk kidney cancer patients who received temsirolimus in this study had 1.6- to 1.7-fold better survival than compared to poor risk patients treated with interferon, by historical control *(54)*. As a result of these observations, a phase III study of temsirolimus was conducted with poor-risk untreated kidney cancer *(55)*. Poor risk is defined by any three of the following features: elevated LDH, anemia, hypercalcemia, time from diagnosis to treatment of less than 1 year, Karnofsky performance status 60–70%, and multiple organ sites of metastasis. In this pivotal trial, 626 patients were randomized to receive either interferon-α escalating to 18 million units three times weekly, temsirolimus 15 mg weekly plus interferon-α 6 million units three times weekly, or temsirolimus 25 mg weekly without interferon. Objective responses were observed in 6% of patients assigned to interferon, 9% of patients assigned to temsirolimus alone, and 11% of patients assigned to combination therapy. The degree of clinical benefit (defined as SD for at least 16 weeks, complete or partial response) was greater in patients who had received temsirolimus or combination therapy than with interferon. Further, progression-free survival was improved with temsirolimus and temsirolimus plus interferon compared with interferon alone (3.7, 3.7, vs. 1.9 months). Patients assigned to temsirolimus 25 mg experienced a 49% improvement in median survival compared to patients treated with interferon alone, (interferon-α—7.3 months vs. temsirolimus 25 mg—10.9 months, $p = 0.0069$). Notably, patients in the temsirolimus plus interferon arm did not show an improvement in median survival compared to interferon alone. These results are particularly important, as no prior study testing medical therapy for metastatic kidney cancer has shown a statistically significant median survival advantage for treatment. Toxicities associated with temsirolimus therapy include stomatitis, rash, fatigue, asthenia, nausea, diarrhea, peripheral edema, vomiting, hyperlipidemia, hyperglycemia, and hypercholesterolemia. The FDA granted approval to temsirolimus in advanced kidney cancer in July 2007.

2.3.2. RAD001

RAD001 is an orally available mTOR inhibitor in development for RCC *(56)*. Results have been presented from 28 patients enrolled in that study. Of the evaluable patients, 36% experienced partial responses and an additional 40% had stable disease greater than 6 months. Median duration of therapy was more than 8 months in this initial report. Based on these data, a large phase III study was undertaken comparing RAD001 to best supportive care in patients who experienced disease progression on sunitinib or sorafenib. This trial is ongoing as are combination studies of RAD001 plus VEGF receptor tyrosine kinase inhibitors.

2.4. Renal Cell Carcinoma—Conclusions

Therapies targeting the critical VEGF receptor in kidney cancer have fundamentally changed the treatment paradigm for this disease. mTOR inhibition in combination with antiVEGF therapy or as monotherapy, also holds significant promise. Because of their marginal activity and high degree of toxicity, immunotherapies have been relegated to second-line status or are not in use at all in this disease. Futher therapeutic trials will seek to optimize the timing, combinations and tolerability of these important new agents.

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45 Advances in the Treatment of Lung Cancer

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CONTENTS

ABSTRACT

Advances in lung cancer research have led to changes in the standard of care. Most of these advances involve management approaches to nonsmall-cell lung cancer (NSCLC). Cure rates for early NSCLC have increased with the use of cisplatin-based postoperative chemotherapy. While chemotherapy continues to be the standard of care in advanced or metastatic disease, the integration of targeted agents has been feasible and has led to increased survival. The currently approved targeted agents include the vascular endothelial growth factor antibody bevacizumab, and the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor erlotinib. Bevacizumab is used in combination with platinum-based chemotherapy in first-line therapy and erlotinib is used as a single agent in relapsed disease. Ongoing trials will evaluate the merit of these drugs in early disease. Inhibitors of multiple other targets are in varying stages of investigation. Increasing awareness of the unique toxicity profile has influenced the eligibility requirements for treatment with these agents. Biomarker discovery has led to the understanding of the biological heterogeneity of tumors and is quickly paving the path for individualized medicine. Such individualized therapy will have impact on both early and advanced disease with regard to treatment with not only targeted agents but also with cytotoxic chemotherapy. These biomarkers include excision repair cross-complementation group 1 (ERCC1), genomic profliling, EGFR protein, mutation, and gene expression. Individualized therapy will maximize the current modest benefit seen with standardized treatments. In small-cell lung cancer, the use of prophylactic cranial radiation has increased survival rates and quality of life in both limited and extensive stage disease. This review will address these recent advances in the treatment of lung cancer.

Key Words: lung neoplasms; chemotherapy; adjuvant; biological markers; angiogenesis; epidermal growth factor receptor; protein kinase inhibitors; monoclonal antibodies; radiotherapy

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1. NONSMALL-CELL LUNG CANCER

1.1. Early Disease

1.1.1. Radiation Therapy

The role of postoperative radiation therapy (PORT) has fluctuated over the past decade. An earlier meta-analysis of PORT in the 1990s demonstrated a reduced risk of local recurrence in stage III (N2) disease without improvement in survival, while there was a detrimental effect on survival with N0–N1 disease *(1)*. Recent retrospective data from the adjuvant chemotherapy ANITA trial and the SEER database while endorsing the negative effect of radiation on survival in patients with N0 and N1 disease, showed improved survival in N2 disease (HR 0.855, 95% CI 0.76–0.95) *(2,3)*.

1.1.2. Adjuvant Chemotherapy

The randomized clinical trials in stages I–III NSCLC which recently led to the standardization of adjuvant chemotherapy are described in Table 1 *(3–7)*. With the exception of the ALPI and the BLT trials, cisplatin-based trials have shown a consistent benefit with adjuvant cisplatin-based chemotherapy. The Cancer and Leukemia Group B (CALGB) 9633 trial was unique by inclusion of only patients with IB tumors and the use of carboplatin. With the exception of tumors ≥ 4 cm (HR 0.66, $p = 0.04$), this study showed no statistically significant survival difference with adjuvant carboplatin-based chemotherapy *(8)*.

The results of the individual randomized trials were reinforced by the LACE (Lung Adjuvant Cisplatin Evaluation) study *(9)*, a meta-analysis of five randomized cisplatin-based trials (ALPI, ANITA, BLT, IALT, and JBR10). This analysis demonstrated a 5-year survival improvement of 5.3% ($p = 0.004$) with adjuvant therapy. Cisplatin plus vinorelbine was found to be marginally better than other drug combinations ($p = 0.04$). In support of subset analysis of the individual trials, the LACE meta-analysis confirmed a nonsignificant benefit of adjuvant chemotherapy in stage IB (HR 0.92, 95% CI 0.78–1.10), a detrimental effect of chemotherapy in stage IA (HR 1.41, 95% CI 0.96–2.09), and a significant survival benefit in stages II and IIIA (HR 0.83, 95% CI 0.73–0.95). In the Canadian JBR10 trial, the benefit of adjuvant chemotherapy was

Table 1
NSCLC Adjuvant Chemotherapy Randomized Trials

Study	*No. of patients*	*Chemotherapy regimen*	*Stages*	*Hazard ratio*	*p value*
ALPI *(6)*	1,206	Cisplatin + mitomycin + vindesine	IA–III	0.96	0.59
BLT *(8)*	381	Cisplatin doublet or triplet	IA–III	1.02	0.90
IALT *(7)*	1,867	Cisplatin doublet	IA–III	0.86	<0.03
NCI-C JBR 10 *(5)*	482	Cisplatin + vinorelbine	1B–II	0.69	0.04
ANITA *(4)*	840	Cisplatin + vinorelbine	IB–IIIA	0.80	0.017
CALGB 9633 *(9)*	344	Carboplatin + paclitaxel	IB	0.80	0.10

ALPI = Adjuvant Lung Project Italy; ANITA = Adjuvant Navelbine International Trialist Association; BLT = Big Lung Trial; CALGB = Cancer and Leukemia Group B; IALT = International Adjuvant Lung Trial; NCI-C = National Cancer Institute of Canada.

observed to extend into elderly patients 66–70 years of age (HR 1.20, 95% CI 0.83–1.73, $p = 0.34$), and 71 to 75 years of age (HR 1.06 95%CI 0.65–1.71$p = 0.83$). However, this overall survival benefit was not seen in those over the age of 75 (HR 2.41 95% CI 1.43 to 4.06 $p < 0.001$)*(10)*.

In summary, cisplatin-based chemotherapy is now the standard of care for resected stages II and III NSCLC. Carboplatin is a reasonable alternative where cisplatin is contraindicated. The benefit of adjuvant chemotherapy in stage IB is unclear although those with larger subset of tumor may derive a benefit. Elderly above the age of 65 years with good performance status should be offered adjuvant chemotherapy while those above the age of 75 require further study. Two ongoing trials will determine the effect of the addition of vascular endothelial growth factor (VEGF) monoclonal antibody bevacizumab and epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) erlotinib to cisplatin-based adjuvant chemotherapy. PORT should be considered for patients with N2 disease at the time of surgery.

1.2. Locally Advanced NSCLC

The optimal therapy for patients with major N2 disease remains controversial. The intergroup 0139 study explored the role of surgery in this stage of disease by randomizing patients with IIIA N2 NSCLC to trimodality neoadjuvant chemoradiotherapy followed by surgery or bimodality definitive chemoradiotherapy*(11)*. Induction chemoradiation consisted of cisplatin/etoposide and 45 Gy of radiation while definitive chemoradiation consisted of the same chemotherapy with radiation to 61 Gy. Although, median progression-free survival (PFS) favored the surgical arm (14 vs. 11.7 months, $p = 0.02$), no significant difference in median survival (MS) was seen (22.1 vs. 21.7 months). Subset analysis showed that the patients on surgical arm who achieved mediastinal lymph node clearance (5-year survival 41% N0 vs. 24% N2) and those who did not undergo pneumonectomy (5-year survival lobectomy 36% vs. pneumonectomy 18%) benefited from surgery after induction therapy. Based on the results of this study, surgery can be recommended to those patients who have achieved mediastinal lymph node clearance after induction therapy or who do not require a pneumonectomy.

For tumors not amenable to surgery (bulky N2 IIIA, and IIIB disease), the superiority of concurrent chemoradiotherapy over sequential chemotherapy and radiation has been established*(12–15)* with improvement in median survivals from 12 to 14 months to 16–26 months. Two recent studies have demonstrated the lack of benefit of consolidation or maintenance therapy after completion of concurrent chemoradiation. In the Hoosier Oncology Group (HOG) study, docetaxel did not improve survival after concurrent cisplatin/etoposide and radiation (MS 21.5 months docetaxel vs. 24.2 months placebo, $p = 0.94$)*(16)*. In the Southwest Onocology Group (SWOG) 0023 study, the incidence of cancer related death was higher in patients treated with maintenance gefitinib after concurrent chemoradiation (MS 23 months gefitinib vs. 35 months placebo, $p = 0.013$) *(17)*. Thus the negative results of these two studies provided valuable lessons on the futility of excessive therapy.

1.3. Advanced Disease

1.3.1. Cytotoxic Chemotherapy

Platinum-based doublet chemotherapy is the backbone of treatment for metastatic NSCLC and provides not only a survival advantage but also an improvement in quality of life*(18,19)*. The recent addition of antiangiogenic agent bevacizumab to cytotoxic chemotherapy has improved platinum-based chemotherapy survival and is described below. It was only recently in 2003 that the American Society of Clinical Oncology guidelines included the use of therapy for relapsed disease*(20)*. Among the cytotoxics, docetaxel and

pemetrexed are both approved for their use in relapsed disease, with median survival of approximately 8 months*(21,22)*. Erlotinib, the epidermal growth factor inhibitor, also used in this setting is described below.

1.4. Targeted Therapies

1.4.1. Epidermal Growth Factor Inhibitors

Erlotinib is the only FDA-approved TKI for treatment of lung cancer in North America, and inhibits phosphorylation of the EGFR TK domain and thus prevents receptor dimerization. In a phase III placebo-controlled randomized trial with previously treated NSCLC patients, erlotinib demonstrated clinical benefit (RR [Response rate risk] 9% erlotinib vs. <1% placebo, $p < 0.001$, MS 6.7 erlotinib vs. 4.7 months placebo HR 0.70; $p < 0.001$)*(23)*. More importantly treatment with erlotinib was associated with improved symptoms and quality of life. Cetuximab a chimeric monoclonal antibody that targets the extracellular domain of the EGFR, is still under investigation. When administered with carboplatin and paclitaxel in a randomized phase II study, median survivals achieved with concurrent and sequential administration of cetuximab with chemotherapy were 11 and 10 months, respectively*(24)*.

1.4.2. Angiogenesis Inhibitors

Bevacizumab, a recombinant humanized monoclonal VEGF antibody is the only FDA-approved antiangiogenic agent in NSCLC. In the ECOG 4599 study, newly diagnosed NSCLC patients were randomized to receive chemotherapy (carboplatin/paclitaxel) with or without bevacizumab (15 mg/kg every 21 days). Response rates (35 vs. 15%, $p < 0.001$), PFS (6.2 vs. 4.5 months HR 0.66; $p < 0.001$, and MS (12.3 vs. 10.3 months, $p = 0.003$) were in favor of the bevacizumab arm. Significant bevacizumab-associated toxicities were hypertension and hemorrhage. Increased pulmonary hemorrhage seen in early bevacizumab studies was associated with squamous histology. For these reasons, in addition to excluding patients with brain metastases and hemoptysis, those with squamous cell carcinoma were excluded in this trial as well. There were 15 treatment-related deaths on the bevacizumab arm due to hemoptysis, febrile neutropenia, and hematemesis*(25)*. The ongoing Avastin in Lung (AVAiL) trial, is investigating different doses (7.5 and 15 mg/kg) of bevacizumab in combination with cisplatin/gemcitabine*(26)*. Preliminary results demonstrate of prolonged PFS with both doses of bevacizumab (HR 0.75, 95% CI 0.62–0.90, $p = 0.002$—low dose; HR 0.82 95% CI 0.68–0.98, $p = 0.03$—high dose) suggesting that a lower dose of bevacizumab may be adequate to provide disease control. Final results are awaited.

In summary the advances in the treatment of advanced NSCLC include the addition of targeted agents bevacizumab and erlotinib. Treatment now includes multiple options including both cytotoxic and targeted therapies. Thus with sequential treatments available, the overall survival of patients with this relentless malignancy has been improved, though noting that further progress is essential.

1.5. Predictive and Prognostic Molecular Markers

Predictive markers provide information about outcome with a specific treatment. In contrast, prognostic factors define overall outcome, independent of treatment, and thus forecast the natural history of the disease.

The lung metagene prognosis (LMP) model, using microarray analysis, was shown to predict the risk of recurrence in early resected NSCLC with 72–90% accuracy. While this model displays prognostic value of an individual's risk for disease recurrence, its predictive ability to define

outcomes with chemotherapy is unknown*(27)*. A future randomized adjuvant clinical trial with LMP-based risk stratification is planned to explore this predictive capability of LMP. Similarly, scientists from Taiwan have identified five genes (DUSP6, MMD, STAT1, ERBB3, and LCK) whose expression in surgically resected specimens was found to be an independent predictor of relapse-free and overall survival with sensitivity and specificity of 98%*(28)*. Excision repair cross-complementation group 1 (ERCC1) enzyme is involved in the nucleotide excision repair pathway. This enzyme system recognizes and removes cisplatin-induced DNA adducts*(29)*, and appears to be a predictive marker for cisplatin resistance. In a retrospective analysis of the IALT adjuvant trial, only patients with ERCC1 negative tumors (HR 0.65, 95% CI 0.5–0.86, $p = 0.002$), but not ERCC1-positive tumors (HR 1.14, 95% CI 0.84–1.55, $p = 0.4$) benefited from cisplatin-based adjuvant therapy*(30)*. Future and ongoing ERCC1-based clinical trials with platinum and nonplatinum therapy will evaluate this marker in a prospective fashion.

The largest category of molecular markers studied are those relevant for the EGFR pathway—EGFR mutation by sequencing, EGFR gene amplification by FISH (fluoresecent in situ hybridization), and EGFR expression by IHC (immunohistochemistry). In the BR.21 study of erlotinib versus placebo, high EGFR *protein expression* was associated with improved response (11 vs. 4%) and longer survival (HR 0.68, $p = 0.02$) with erlotinib in univariate analysis*(31)*. In the IDEAL (phase II trials of single-agent gefitinib) and INTACT trials (phase III trials of chemotherapy with or without gefitinib), the RR to gefitinib were higher in patients with the *mutation* (46% mutant vs. 10%, wild type $p = 0.005$)*(32)*. However, in terms of survival outcome, the mutation predicted for a better survival irrespective of the treatment administered (HR 0.48, 95% CI 0.29–0.82). Thus EGFR mutation may be a prognostic indicator for survival rather than a predictor of outcome to treatment. Gene *amplification* in the INTACT trials exhibited prognostic abilities similar to the EGFR mutation. Irrespective of treatment, survivals were >20 months in patients with amplification versus 10.2 months in patients without amplification (HR 0.46, 95% CI 0.25–0.83)*(32)*. But the ISEL and the BR21 studies supported predictive capabilities of this marker. In the ISEL trial, survival with gefitinib was better in those with gene amplification (MS gefitinib: 8.3 months vs. placebo: 4.5 months, HR 0.61, 95% CI 0.36–1.04, $p = 0.06$)*(33)*. Those with low gene copy number had a worse survival with gefitinib (MS gefitinib: 4.3 months vs. placebo 6.2 months, HR 1.16, 95% CI 0.81–1.64, $p = 0.417$). Similar to the ISEL study, univariate analysis of the BR21 study associated EGFR gene amplification with better survival in patients treated with erlotinib (HR:0.44, 95% CI 0.23–0.82, $p = 0.008$), but this benefit was not seen in multivariate analysis*(31)*.

In summary, studies so far have elucidated predictive or prognostic capabilities of biomarkers detected in patients with NSCLC. Lack of standardization of technical methods may be a contributor to inconsistencies in results seen across trials. Currently, targeted agents have been used in the unselected NSCLC patients, but with biomarker-based stratification, ongoing trials will identify patients most likely to experience a differential benefit from targeted agents.

2. SMALL-CELL LUNG CANCER

Platinum based chemotherapy with early concurrent thoracic radiation therapy for suitable candidates*(34–36)* remains the backbone of treatment of early disease. Prophylactic cranial irradiation (PCI) is recommended for those who have achieved a complete or near complete response*(37)*. In extensive disease, one recent trial is worthy of discussion. Patients with response (partial or complete) to chemotherapy were randomized to receive PCI versus none*(38)*. PCI reduced 1-year cumulative risk of brain metastasis (14.6%, 95% CI 8.3–20.9 vs. 40.4%, 95% CI

32.1–48.6), and significantly prolonged PFS (HR = 0.76, CI 0.59–0.96, $p = 0.0218$), and OS (1-year survival, 27.1 vs. 13.3%, $p = 0.0033$), suggesting that PCI should now be considered for patients with extensive disease who have had initial response to chemotherapy.

3. CONCLUSIONS

The most significant advances in NSCLC have been in early and advanced disease. In early disease adjuvant cisplatin-based chemotherapy has improved outcomes. The availability of multiple well-tolerated cytotoxic regimens has made sequential treatment of advanced disease possible with survival improvement. Targeted agents have been added to the arsenal against lung cancer. Understanding the mechanism of targeted agents enables the utilization of such agents to their maximum potential. For example, monoclonal antibodies supplement the benefit of cytotoxic agents while TKIs are best used as single agents. Retrospective studies have provided insight into the predictive powers of molecular markers. Increasing awareness of the biological heterogeneity of tumors and the incorporation of such biomarkers into prospective clinical trials will create a paradigm shift toward individualized therapy.

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XIII Recent Advances in the Treatment of Melanoma

46 Pegylated Interferons in the Adjuvant Treatment of Melanoma

Dirk Schadendorf, MD

CONTENTS

ABSTRACT

Melanoma in its advanced stage is highly therapy resistant. Interferons are standard for adjuvant treatment in patients with high-risk melanoma. High- and low-dose interferon regimes have reproducibly shown to prolong relapse-free survival. The new generation of pegylated interferon is hoped to improved clinical efficacy. However, a first randomized clinical phase III study (EORTC 18991) conducted by the EORTC melanoma group comparing 5 years of PEG-Intron versus observation in stage III (N1 and N2) melanoma patients did not prolong overall survival in the intent-to-treat analysis. Clinical benefits were most prominent in the N1 population and patients with ulcerated primaries. In the N2 patients with palpable lymph node metastases no interferon-induced benefit was seen. As long as outcomes in the treatment of advanced metastatic melanoma remain poor, patients should be recruited into well-designed clinical trials.

Key Words: relapse-free survival; overall survival; melanoma; IFN effects; AJCC stage III

1. INTRODUCTION

Cutaneous melanoma is the most frequent cause of mortality from skin cancers. Depending on age, gender, anatomical site, and tumor thickness 20–25% of all primary melanoma will spread. Once melanoma has spread to regional lymph nodes (stage III disease), survival rates drop to

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approximately 30–55% after 5 years. Dissemination to distant visceral organs will render those patients not curable with a median survival time in stage IV of approximately 6–12 months *(1)*. This is mainly because no treatment in stage IV melanoma has to date demonstrated any survival benefit. Therefore, treatment as early as possible in the clinical course of disease should be able to reduce the risk of disease progression. In the adjuvant setting interferons have been used over the last three decades with limited success. This chapter will briefly review the current status of adjuvant therapy using interferon-α in melanoma and newest developments.

2. BACKGROUND AND HISTORY OF INTERFERONS

Since the 1980s the interferons play an important role in the treatment of high-risk primary and of metastatic melanoma *(2,3)*. Different types of interferon have been studied and applied to melanoma patients *(4–6)*. Besides recombinant interferon-α, interferon-β and interferon-γ as well as natural interferon mixtures have been tested in melanoma patients with limited clinical benefit. Twenty-one different interferon-α genes are located on chromosome 9q21 in humans *(7)*. Whether these different subtypes of interferon-α exhibit differential functionality is presently unclear, because amino acid sequences are in part highly homologous. Two recombinant human interferons have been registered for clinical use in melanoma: interferon-α-2a (Roferon-A®) and interferon-α-2b (Intron-A®). The difference between those two interferons is small with only 2 of 166 amino acid changes leading to indistinguishable therapeutic and adverse effects of both cytokines.

Interferons-α in stage IV melanoma has achieved objective response rates of 10–15% in smaller mostly monocentric studies *(8,9)*. A recently published study using pegylated interferon in metastatic melanoma using three different dosages reported response rates in the range *(10)*.

3. EFFECTS OF INTERFERON-α

Interferon-α and interferon-β both belong to type I-interferons and bind to interferon type 1 receptor *(7,12)*. The signaling cascade initiated by receptor binding of interferon-α is well characterized *(12–14)*. Binding of interferon-α to the receptor activates two janus kinases tyk-2 and JAK-1 (Fig. 1 and Color Plate 45). Activated janus kinases recruit "STAT" factors in the cytoplasma. Six different "STAT" factors are currently known which form dimers that bind specifically DNA sequences of so-called interferon-responsive genes in the nucleus acting as transcription factors (Fig. 1). The variety of interferon-responsive genes is large and include those for virus inhibition like the MX proteins (inhibiting viral replication), 2′5′-OAS (induces mRNA degradation) and PKR (inhibiting translation). Additional genes are involved in immune regulation like the activation of IL-12, IL-15, and interferon-γ. Likewise toll-like receptors are activated by interferon-α. Growth inhibition of tumor and virally infected cells is also mediated by interferons (Fig. 2).

4. PEGYLATED INTERFERON-α COMPARED TO CLASSIC INTERFERON-α

Recombinant interferon-α as produced by biotechnology in *Escherichia coli* are proteins without any biochemical modifications. Polyethylene glycol (PEG) modification can be done chemically in various forms leading to molecules with several side chains that considerably influences the pharmacokinetics and the half-life time of those molecules. The half-life time in the serum ranges between 3 and 6 h for the classic interferon-α compared to 40–60 h for the pegylated interferon-α depending on the exact PEG modification *(15,16)*.

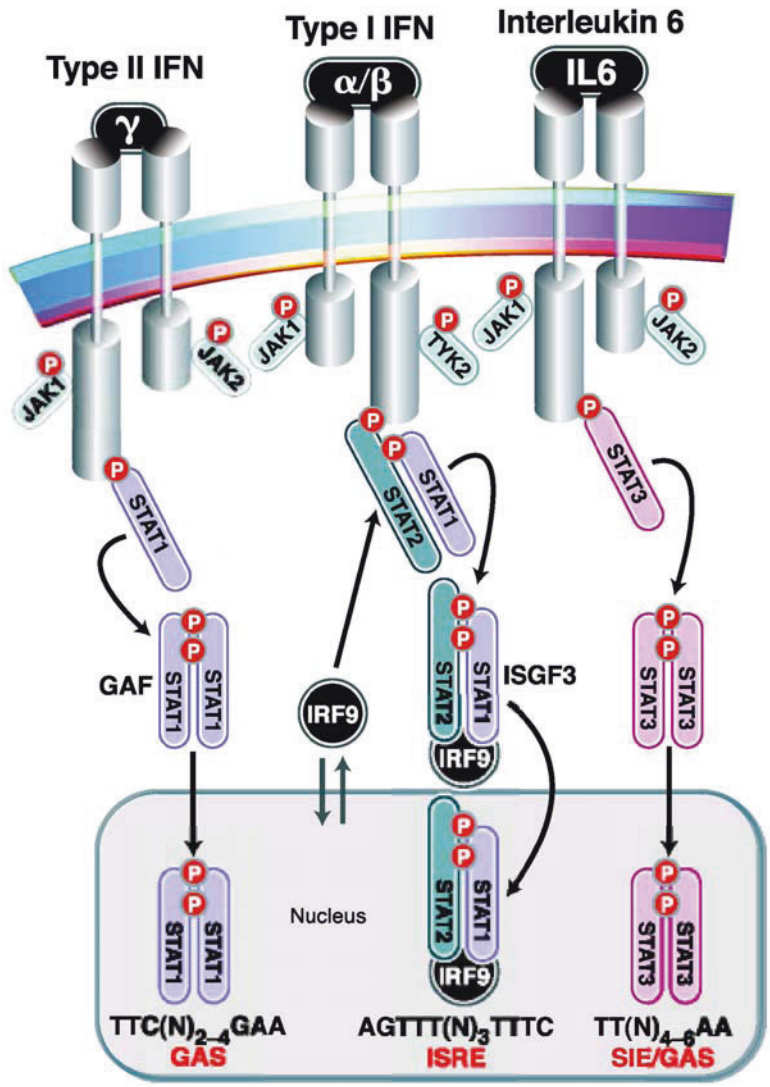

Fig. 1. Interferon (IFN) signal transduction. IFNs bind to specific receptors and induce tyrosine phosphorylation of receptors by JAK kinases providing a docking site for the STAT proteins which are subsequently phosphorylated by the Jak kinases. Activated STAT proteins dimerize and translocate into the nucleus in order to directly bind to specific DNA sequences as transcription factors (from *[11]*). (*see* Color Plate 45)

But not only pharmakokinetics and half-life is changed by PEG modification, also elimination is shifted from renal to hepatic excretion. The extended half-life of pegylated interferon-α allows a once weekly application schedule instead of three times weekly application of classic interferon-α accompanied by long-lasting interferon levels in the circulation leading to improved activity in hepatitis virus elimination *(17–22)*. Biological activity of classic interferon- α compared to pegylated interferons seemed not to differ to much as demonstrated by recent gene profiling experiments reported *(23,24)*. In oncology, there are first hints that pegylated interferon-α may be more effective in the treatment of malignancies such as in renal cell cancer and chronic lymphatic leukemia compared to classic interferons *(25–27)*.

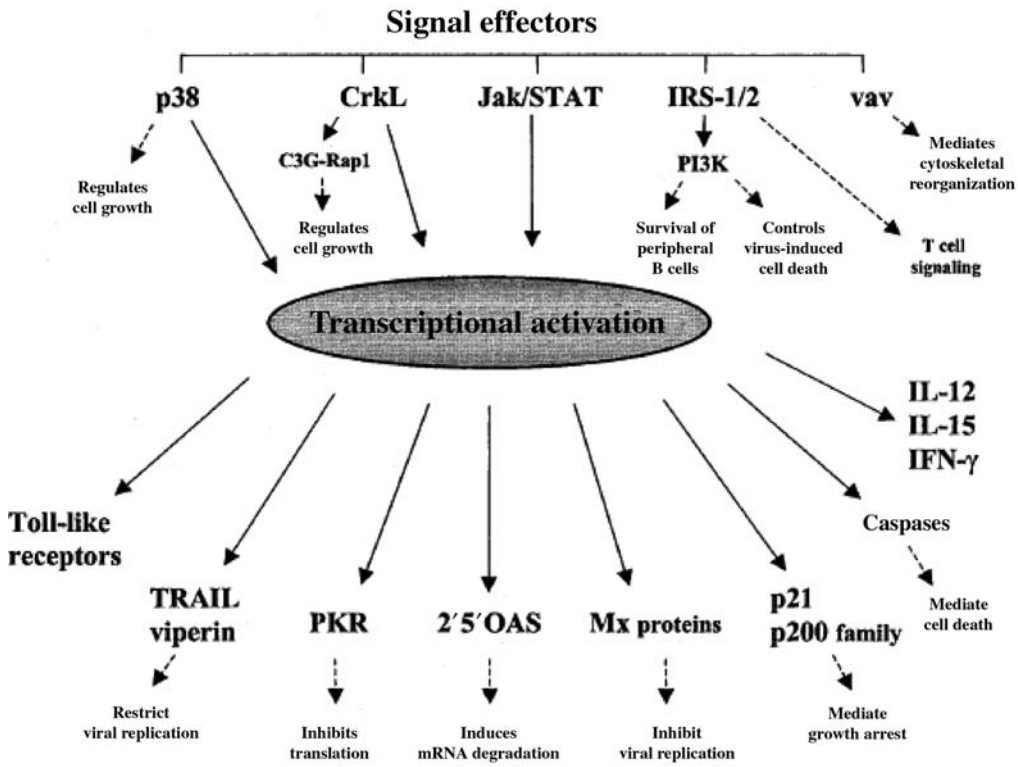

Fig. 2. Summary of IFN effects. IFNs exhibit a large variety of biological effects dependent on cell type, differentiation stage and IFN concentration ranging from growth arrest, immune acitivation via direct and indirect interaction, inhibition of viral replication and protein translation.

5. ADJUVANT TREATMENT WITH INTERFERON-α

Classic interferon-α has shown over the last two decades some beneficial effects in the adjuvant treatment of high-risk cutaneous melanoma patients. Since no other drug so far has shown comparable clinical effects classic interferon-α is well established in the adjuvant treatment in clinical stages II and III, although no survival benefit for melanoma patients under treatment with interferon-α could convincingly be demonstrated. Mainly two different treatment regimens have been introduced with high and low dosages of interferon-α. Significant benefits in terms of recurrence-free and overall survival have been shown for both regimens in single trials *(28–32)*. However, some studies were unable to demonstrate those beneficial effects *(33–37)*. Recent meta-analysis of all available interferon data in melanoma detected a highly significant impact on relapse-free survival *(38)* (Fig. 3).

For the first time also a small, but statistically significant improvement of overall survival in the range of 3% for the entire study population of more than 6,000 patients analysed was detectable *(39)*. Dosage and duration of treatment seemed to be of less importance for the small clinical benefits *(37–41)*.

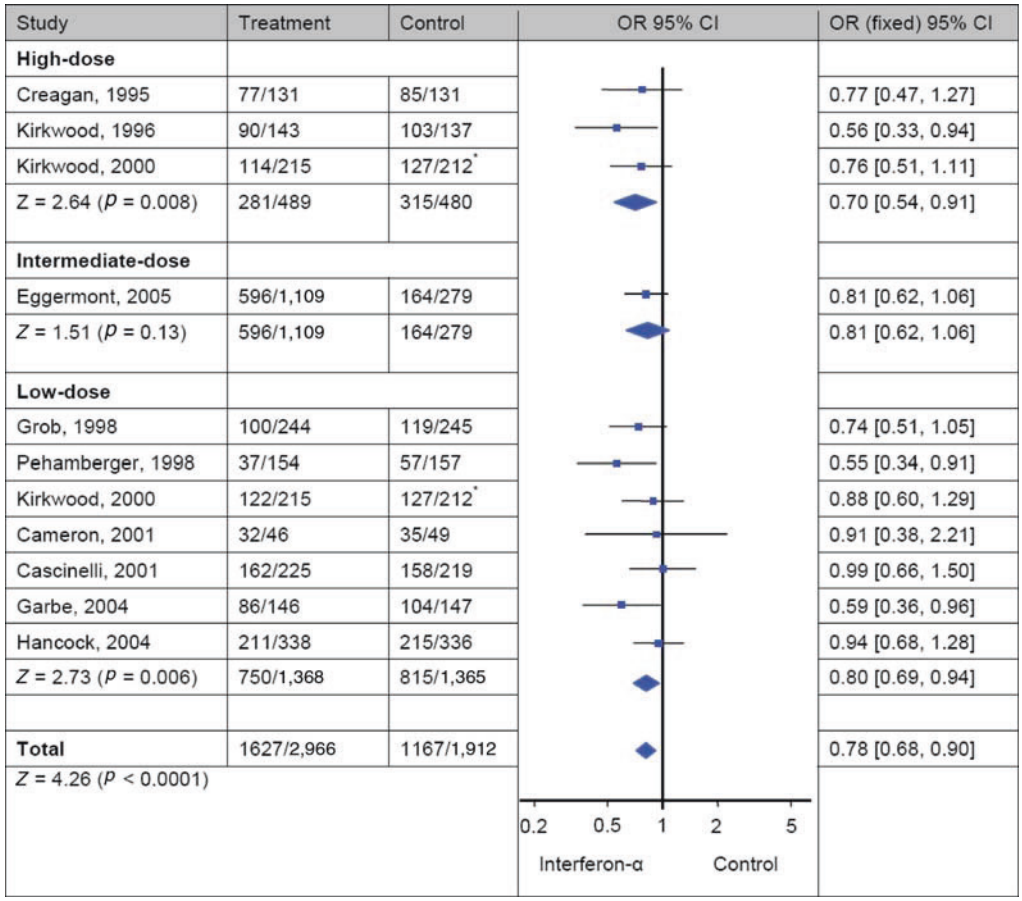

Fig. 3. Meta-analysis of adjuvant IFN treatment. IFN given at different durations and various concentrations leads to a strong impact on relapse-free survival in almost 5,000 patients treated in clinical trials compared to observation (from *[38]*).

6. ADJUVANT TREATMENT WITH PEGYLATED INTERFERON-α

Currently, for adjuvant treatment with pegylated interferon-α in high-risk melanoma patients several clinical trials have been designed and have closed recruitment in the last year. However, only one large trial EORTC 18991 performed by the EORTC melanoma group has been so far completely evaluated and was first reported at ASCO 2007.

Another trial has been initiated by the European Association of Dermatologic Oncology (EADO) lead by C. Garbe (Germany), H. Pehamberger (Austria), and B. Delaunay (France). In this trial, patients with stage II-melanoma, according to the "old" TNM-classification, have been recruited. This comprises patients with more than 1.5-mm tumor thickness, including those, who have been found positive for micrometastatic disease by sentinel lymph node biopsy. The trial recruited 890 patients which were randomized into the following arms: Pegylated interferon-α2b 100 μg once weekly for 36 months, versus reference treatment with low-dose interferon-α2b 3 × 3 MIU/weekly for 18 months which is an approved dosing regimen in Europe. Recruitment goals were reached in June 2005. The study is powered to detect a 10% improvement of disease-free survival at 5 years as the primary study endpoint.

A third trial led by C. Garbe has been initiated by the German Dermatologic Cooperative Oncology Group (DeCOG) in which pegylated interferon-α2a (Pegasys®) has been tested. The trial has recruited 880 melanoma patients in stage IIA–IIIB (Table 1A, B) between October 2004 and May 2007. Patients were randomized into two arms: Treatment with 180 μg pegylated interferon-α2a (Pegasys®) weekly for 24 months, versus treatment with classic interferon-α-2a, (Roferon-A®) 3 × 3 MIU weekly for 24 months. The study was powered to detect a 10% improvement of distant metastasis-free survival at 5 years.

Table 1A
Clinical Staging of Cutaneous Melanoma (ASCC 2002)

	Clinical staging 1			*Pathological staging 2*		
	T	*N*	*M*	*T*	*N*	*M*
Q	Tis	N0	M0	Tis	N0	M0
IA	T1A	N0	M0	T1a	N0	M0
IB	T1b	N0	M0	T1b	N0	M0
	T2a	N0	M0	T2a	N0	M0
IIA	T2b	N0	M0	T2b	N0	M0
	T3a	N0	M0	T3a	N0	M0
IIB	T3b	N0	M0	T3b	N0	M0
	T4a	N0	M0	T4a	N0	M0
IIC	T4b	N0	M0	T4b	N0	M0
III3	Any T	N0	M0			
		N2				
		N3				
IIIA				T1-4a	N1a	M0
				T1-4a	N2a	M0
IIIB				T1-4a	N1a	M0
				T1-4a	N2a	M0
				T1-4a	N1b	M0
				T1-4a	N2b	M0
				T1-4a/ab	N2c	M0
IIIC				T1-4b	N1b	M0
				T1-4b	N2b	M0
				Any T	N3	M0
IV	Any T	Any N	Any M1	Any T	Any N	Any M1

Table 1B
N Classification of Stage III Melanoma in detail (AGCC 2002)

N classification	*No. of metastatic nodes*	*Nodal metastatic mass*
N1	1 node	a: micrometastasis *(1)*
		b: macrometastasis *(2)*
N2	2–3 nodes	a: micrometastasis *(1)*
		b: macrometastasis *(2)*

(*Continued*)

Table 1B
(Continued)

N classification	*No. of metastatic nodes*	*Nodal metastatic mass*
		c: in transit met(s)/satellite(s) without metastatic nodes
N3	Four or more metastatic nodes, or matter nodes, or in transit met(s)/ satellite(s) with metastatic nodes	

Thus, the future will show if pegylated interferon-α is effective as an adjuvant treatment at all and if it is more effective than classic nonpegylated interferons. For the EADO as well as the DeCOG trial several more years observation are needed to judge whether the study goals will be reached. However, trial results of EORTC 18991—as the first trial of pegylated interferons in adjuvant melanoma treatment have recently been published at ASCO 2007 *(42)*. Into this EORTC 18991 trial 1,256 patients with microscopic and macroscopic lymph node metastasis were recruited between July 2000 and August 2003 *(43)*. The trial design was simple and patients were equally randomized into the following arms: Patients in Arm A were treated with pegylated interferon-α2a (PEG-Intron ®) 6.0 μg/kg per week for the first 8 weeks followed by a long-term maintenance treatment period of 5 years at 3.0 μg/kg per week. Patients in Arm B were closely followed only. The study was aimed to detect a 24% risk reduction with 90% power based on the assumption of a 40% distant metastasis-free survival rate in the control arm at 4 years. Because of the pivotal character of this clinical trial FDA requested a change in the primary study endpoint from distant metastasis survival to relapse-free survival before any statistical evaluation was done.

Distribution of patients into both arms was highly homogeneous as one would expect using an accurate randomization procedure and adequate stratification factors. Interestingly, induction phase was completed in almost all patients over 8 weeks; however, maintenance treatment reached only duration of 15 months in median. The main reason in around 50% of patients for this was disease progression. In one third of the patients toxicity issues led to treatment discontinuation resulting in only 23% of melanoma patients staying on treatment with pegylated interferon-alpha 2b at year 4–5. Main toxicities grade 3/4 which were observed were liver toxicity (10%), fatigue (15%), and depression (5%).

From the regulatory endpoint, relapse-free survival time was significantly prolonged from 25.5 months in the observation arm with a 4-year survival rate of 38.9% compared to 34.8 months and a 45.6% survival rate at 4 years in the PEG-treated patient cohort ($p = 0.011$) on an intent-to-treat (ITT) analysis. Distant metastasis-free survival which was originally chosen as the primary study endpoint differed also between both arms with 36.1 months median (4-year rate: 45.4%) in the observation group and 45.6 months in the treated cohort (4-year rate: 48.2%) missing statistical significance slightly ($p = 0.107$). Disappointingly, overall survival in the ITT analysis was completely overlapping in both groups for the entire study population. Nevertheless, because of the large size of the study and the well-balanced distribution additional analyses were performed taking into account tumor load (N1, microscopic tumor invasion versus N2, more than 1 node involved), and ulceration of the primary tumor as known risk factors.

Hazard ratio over time for N2 patients did show only a small benefit for relapse-free survival (around 12%), but no effects for distant metastasis-free survival and overall survival comparing

observation and treatment group. In contrast in patients with micrometastasis (N1) a clear treatment benefit of pegylated interferon could be demonstrated. Hazard ratios of relapse-free survival and distant metastasis-free survival were improved in 27 and 25% of patients, respectively. Overall survival benefit was smaller at 12% benefit. The hazard ratio curve over time was always below the observation curve strongly suggesting an increased cure rate in this patient population. *(44)*

Interestingly, the ulceration status of the primary cutaneous melanoma had a strong predictive impact on the treatment benefit with pegylated interferons despite the fact that only patients with lymph node involvement were included in EORTC 18991. In the entire population of 373 patients with ulcerated primary the beneficial effect of treatment with pegylated interferon was visible from the very beginning; this effect was even more pronounced in N1 patient population.

In conclusion, effects of interferon in the adjuvant therapy of melanoma are highly consistent in modern times of melanoma study conduct. Relapse-free survival can reproducibly be prolonged independent of duration of treatment. Effects on distant metastasis survival and overall survival remain small and only subgroups of melanoma patients seem to benefit. Recent reported EORTC 18952 data *(45)* and EORTC 18991 data with more than 2,500 patients in total argue for a benefit induced by interferons based on tumor load (IIa vs. N1 vs. N2). Furthermore, ulceration of the primary melanoma seems to indicate a particular sensitivity to the benefits of an interferon treatment. Whether this observation holds true will be tested in a subsequent EORTC trial which will start in 2009. For patients which a higher risk profile such as N2 and N3 patients alternative therapeutic approaches other than interferon need to be explored. CTLA-4 antibodies are here a prime candidate.

As long as outcomes in the treatment of advanced metastatic melanoma remain poor, patients should be recruited into well-designed clinical trials.

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47 Sorafenib, a Multikinase Inhibitor: Results from Clinical Trials in Melanoma Patients

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Contents

ABSTRACT

The prognosis of metastatic melanoma is poor with a median survival of 6–9 months. Remission rates after systemic treatment range from 5 to 20%, but there is no clear effect on overall survival. The single-agent chemotherapy with dacarbazine (DTIC) is often used as a reference agent in prospective randomized clinical trials, although a striking effect on overall survival has never been shown. Thus, there is an ongoing debate about the appropriate medical treatment for patients with advanced metastatic melanoma (AJCC stage IV).

Sorafenib is a multikinase inhibitor, which specifically blocks molecular targets of the signal transduction pathway in cancer cells. Furthermore, its antiangiogenic potential is well described. Sorafenib is successfully used in advanced renal cell cancer and is one of the first new agents with molecular targets, which was brought into phase II and phase III trials in metastatic melanoma. However, as a single-agent therapy, sorafenib seems to be of limited use only. Also the combination of sorafenib with chemotherapeutic agents has failed to show striking effects on progression free and overall survival in smaller phase II and phase III trials. Nevertheless, these findings need

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to be confirmed in large-sized trials before final conclusions can be drawn. Thus, there is a consensus that patients with disseminated malignant melanoma shall be recruited into clinical trials as a treatment of first choice.

Key Words: Melanoma; metastasis; sorafenib; multikinase inhibitors

1. INTRODUCTION

Cutaneous malignant melanoma is the cause of the majority of deaths from skin cancer. Still there is a rise in the incidence and mortality of melanoma in Caucasians. Whereas the prognosis of patients with primary tumors and negative sentinel nodes is excellent in most cases, it is less favorable in patients with sentinel nodes containing micrometastases, in those with macrometastases in the lymph nodes or skin and particularly in patients with advanced metastatic melanoma confined to visceral organs. The prognosis varies from 60% 5-year survival in sentinel node-positive patients (stage IIIA) to less than 10% for patients with stage IV disease according to the AJCC classification system *(1)*.

The appropriate systemic treatment for advanced metastatic melanoma patients is still controversially discussed. A single-agent chemotherapy for instance with dacarbazine (DTIC) is far away from being accepted as a gold standard, but DTIC can be considered as a reference drug and an appropriate comparator in clinical trials. The response rates for DTIC in recently conducted phase III trials are in a range from 7 to 12% only.

A systematic review of 41 randomized clinical trials identified by a comprehensive search demonstrated an increase of response rates for combinational chemotherapy and cytokine approaches, but however, no prolongation of the overall survival time *(2)*. The conclusion of the authors is that patients with stage IV melanoma should preferentially be treated within controlled clinical trials so that the potential benefits of new treatment concepts can be thoroughly examined. It is unlikely that a further development of new cytotoxic drugs is likely to effect the benefits for melanoma patients substantially. Therefore, other strategies have been developed in the last decade.

Among these, the specific blockade of molecular targets, especially of the signal transduction pathway in melanoma cells, seems to be an attractive new approach in attacking melanoma cells. Sorafenib was the first agent brought into large phase II and phase III trials in metastatic melanoma. The mode of action of this multikinase inhibitor and the potential benefits for metastasized melanoma patients will be discussed in detail.

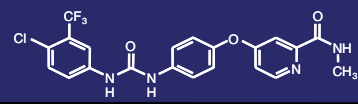
2. SORAFENIB: MODE OF ACTION

The Ras–Raf–MEK–ERK signaling pathway is activated in the vast majority of melanomas. Activation occurs through either N-ras or B-Raf mutations, both of which arise early during melanoma pathogenesis and are preserved throughout tumor progression *(3)*. B-Raf or N-ras gene mutations have been found to be mutated in 69 and 11% of melanoma cell lines, respectively *(4)*. Another study reported that melanoma cell lines which had neither B-Raf nor N-ras mutations had high levels of ERK activation *(5)*. Therefore, it appears that most melanomas activate the Ras–Raf–MEK–ERK pathway by various mechanisms.

Sorafenib (former name: BAY 43-9006) is a potent inhibitor of Raf kinase in vitro and in vivo. Significant dose-dependent antitumor activities in several human tumor xenografts models have been demonstrated. Additionally, sorafenib inhibits several other receptor

Table 1
Potential Targets for Sorafenib and Inhibition Concentrations

Sorafenib, a multikinase inhibitor



Kinase assays	IC_{50}
C-Raf	2 nM
mVEGFR-2, VEGFR-3	6–10 nM
wt B-RAF, V600E *b-Raf*	20–40 nM
p38, PDGFR-β	28–38 nM
Flt-3, c-KIT	40–80 nM
EGFR, MEK, ERK	Inactive at 10 μM

Adapted from Wilhelm et al., Cancer Res. 2004;64(19):7099–7109

tyrosine kinases that are involved in the tumor progression. For instance, sorafenib is a potent inhibitor of vascular endothelial growth factor (VEGF) receptor 2 and 3 in vitro. Anticancer activity was observed in tumors that have Ras mutations as well as in cancers without Ras mutations. These suggest a potential use of this component in a larger spectrum of malignancies with a variety of molecular etiologies, all of which have not yet been definitively defined. The inhibition of molecules related to the signal transduction and angiogenesis in tumor cells is thus leading to antiproliferative effects as well as antiangiogenic effects in vitro and in vivo.

Table 1 shows potential targets for sorafenib as a multikinase inhibitor as well as the inhibition concentrations.

3. SORAFENIB: CLINICAL TRIALS IN VARIOUS CANCERS

The vast majority of clinical data does exist for patients with advanced renal cell cancer (RCC), where sorafenib as an oral multikinase inhibitor got an approval by the FDA and EMEA recently. It has been demonstrated that sorafenib is significantly effecting the relapse-free survival time and very recently it was shown that it effects also the overall survival time *(6,7)*.

At ASCO 2007, the data on the first randomized, controlled clinical trial of sorafenib in hepatocellular cancer (HCC) were released. The *S*orafenib *H*CC *A*ssessment *R*andomized *P*rotocol (SHARP) demonstrated that sorafenib extends the overall survival by 44% (hazard ratio = 0.69, $p = 0.0006$) versus placebo alone. There were no meaningful differences in serious adverse events rates between the sorafenib- and placebo-treated groups. Typical adverse events related to sorafenib were diarrhea, rashes (Fig. 1 and Color Plate 46), and hand–foot skin reactions (Fig. 2 and Color Plate 47) well known from other clinical trials with this agent *(8)*.

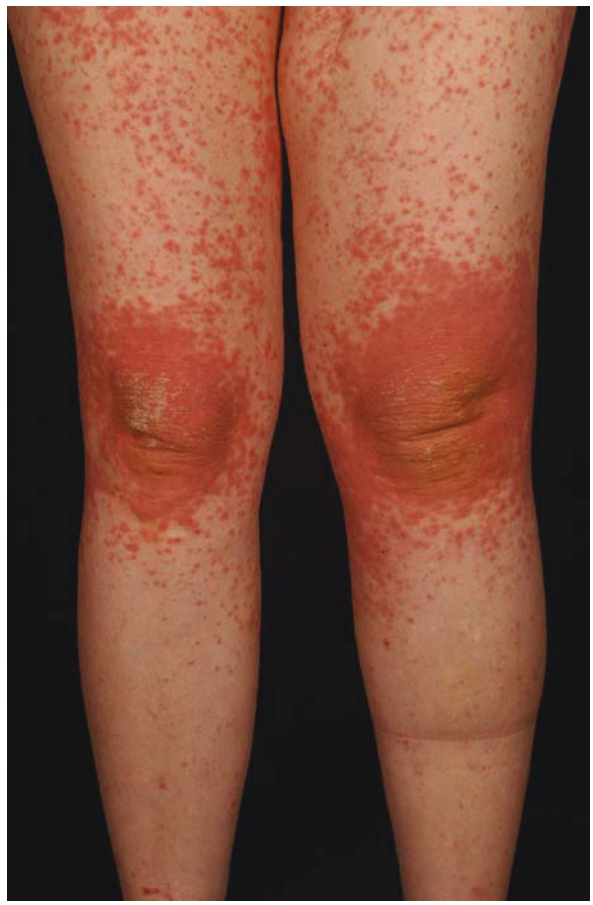


Fig. 1. Typical papular rash in a patient treated with sorafenib. (*see* Color Plate 46)

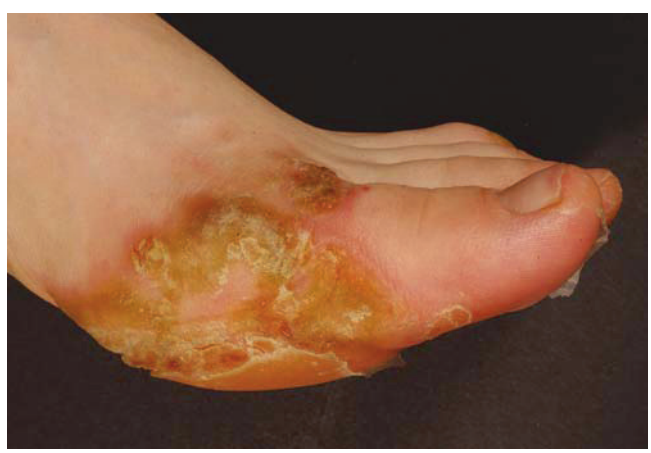


Fig. 2. Typical hyperkeratotic hand–foot syndrome in a patient treated with sorafenib. (*see* Color Plate 47)

4. SINGLE-AGENT SORAFENIB: CLINICAL TRIALS IN MELANOMA

As part of a larger randomized discontinuation phase II trial 39 patients with advanced melanoma were treated with single-agent sorafenib at four international sites. Seven patients had a stable disease at 12 weeks as their best response indicating that there is a limited activity of sorafenib as a single-agent treatment against melanoma. The median progression-free survival (PFS) was 11 weeks *(9)*. Another uncontrolled clinical and biomarker trial on 19 melanoma patients of whom 11 were evaluable for response demonstrated two partial responses and three stable diseases as best objective response. One patient with a partial response (PR) had a wild-type B-Raf, the other patient with PR a mutated B-Raf. The biomarker study was not able to draw a final conclusion on the relationship between the B-Raf mutational status and clinical responses *(10)*.

5. SORAFENIB PLUS DACARBAZINE IN MELANOMA

The rationale to combine DTIC with sorafenib is that DTIC treatment had been demonstrated to upregulate VEGF expression in melanoma cells in vitro *(11)*.

In vivo models indicated that these cells had increased tumor growth and metastatic potential *(12)*. Subsequently, a phase I study combining these agents showed that they the combination was well tolerated *(13)*. Two phase II trials have been conducted in the combinational setting. In 32 melanoma patients the disease control rate (CR + PR + SD) was 65% including 12.5% of patients with a partial response *(14)*. The PFS time was 3.5 months, the median overall survival 9.3 months. In a second study, a randomized, phase II design for sorafenib plus DTIC has been chosen. The study was conducted in the USA and the preliminary data have been released at ASCO 2007. McDermott and coworkers *(15)* treated unresectable stage III and stage IV patients with an ECOG performance status of either 0 or 1. In group A, patients received DTIC (1,000 mg/m^2) intravenously every 21 days combined with sorafenib (400 mg) bidaily on days 1–21. In group B, patients received the same schedule for DTIC plus two tablets of a placebo from day 1 to 21. 98 patients had been included within a 12-month recruitment period. The primary endpoint of this trial was PFS. The randomized phase II trial demonstrated 12% of partial responses in the DTIC plus placebo arm in contrast to 24% in the DTIC plus sorafenib arm. The number of stabilizations was equally distributed between the two arms. Of note, all responses have been confirmed by an independent radiological review committee. The PFS showed a median of 82 days for DTIC plus placebo and 148 days for DTIC plus sorafenib, respectively. The PFS rate at day 180 was 18% in the DTIC/placebo arm in contrast to 41% in the DTIC/sorafenib arm *(15)*. There is still an open discussion whether the producing pharmaceutical companies are proceeding with a randomized phase III trial in this setting to confirm the preliminary phase II data.

6. SORAFENIB PLUS TEMOZOLOMIDE IN MELANOMA

In a relatively complex phase II study with four different treatment arms, a combination of sorafenib and temozolomide has been tested in advanced metastatic melanoma patients *(16)*. Patients received sorafenib 400 mg bidaily per os as a single agent for 1 week, followed by a combination therapy of sorafenib and either an extended dose scheduling for temozolomide (75 mg/m^2, days 1–7, per os, once daily, first 6 out of 8 weeks) or standard dose temozolomide (150 mg/m^2, days 1–5, per os, every 4 weeks). Patients with and without brain metastases have been treated. The trial was stratified for patients with and without prior temozolomide therapy, too. While no complete responses have been observed, the trial demonstrated a relatively encouraging rate of partial responses between 17 and 39% in a preliminary analysis. Only the

patients with prior temozolomide treatment had a poor response rate (4%). Surprisingly, patients with brain metastases from melanoma did relatively good with a response rate of 17% and a PFS of 5.7 months. However, the final analysis of this four-arm phase II trial needs to be released in detail before final conclusions could be drawn on this new combination scheme.

7. CARBOPLATIN, PACLITAXEL WITH OR WITHOUT SORAFENIB

An oligocentric, phase I/II, open-label study on the combination of carboplatin (AUC 6) and paclitaxel (225 mg/m^{2}day) each on day 1 every 3 weeks combined with sorafenib (400 mg bidaily per os on days 2–21) was reported recently. A total of 105 patients have been treated. The disease control rate (CR + PR + SD) was 85% with only 5% of patients with progressive disease after the first evaluation and another 10% of patients with a "nonassessable disease." However, the responses were investigator-assessed and not controlled by an independent radiological committee. In all, 26% of the patients had a partial response, 1% were complete responders, and 58% patients demonstrated stabilized disease. The median PFS time was 8.8 months with significant differences for responders (15.2 months) compared to patients with stable disease (6.7 months). The majority of patients suffered from hematologic toxicity, mainly neutropenia and thrombocytopenia of CTC grades 3 and 4. Typical side effects of sorafenib in these combinational settings were rashes (Fig. 1), the development of a hand–foot syndrome (Fig. 2) and diarrhea.

Of interest, it was noted that the B-Raf mutational status of the melanoma patients did not correlate to the clinical outcome of the patients *(17)*.

The promising results of these regimens were leading to a phase III trial on carboplatin and paclitaxel with or without sorafenib (PRISM) in second line for pretreated stage IV melanoma patients. This placebo-controlled, double-blinded trial was conducted in 271 patients and the analysis was presented at ASCO 2007 *(18)*. The schedule and dosages used were the same as in the phase I/II trial. The primary endpoint was PFS, the secondary endpoints contained overall survival, tumor response rates and the duration of response. An independent radiological review of the computed tomography scans revealed a partial response rate of 11% for carboplatin/paclitaxel plus placebo and 12% for carboplatin/paclitaxel plus sorafenib. There were neither differences in the number of patients with stable disease nor for PFS (17.9 weeks in the placebo-containing arm and 17.4 weeks in the sorafenib-containing arm). The PFS rate at 180 days was 29% in the placebo-containing arm in contrast to 32% in the sorafenib-containing arm *(18)*.

The study results were surprising since the combination of the cytotoxic agents paclitaxel and carboplatin has never produced such an encouraging PFS rate before, particularly because this phenomenon was observed in refractory melanoma patients.

A first-line phase III trial with a similar design as the PRISM study conducted by a US intergroup is ongoing. This study has a sample size of 800 patients. Here the primary endpoint is overall survival.

8. OTHER PHASE II TRIALS WITH SORAFENIB

A number of phase II trials of sorafenib in combination with other agents have been initiated. The homepage of the National Cancer Institute (NCI) provides an overview on registered trials under www.clinicaltrials.gov.

In 2007, three clinical trials on the combination of sorafenib and conventional interferon-α have been published *(19–21)*. A phase I trial in patients with unresectable and/or metastatic renal cell carcinoma and melanoma showed that a combination of sorafenib (maximum doses of 400 mg twice daily) with interferon-α (maximum dose of 9 MIU thrice weekly) demonstrated to

Table 2
Overview on Randomized Clinical Trials with Sorafenib in Melanoma

Author	*Drug(s)*	*Phase*	*BRR*	*Median PFS*	*6-month PFS*	*OS*	*Comments*
McDermott et al. *(15)*	DTIC + sorafenib	II	PR: 24% DCR: 71%	21.1 weeks	41%	45.6 weeks	• no prior chemotherapy • one prior immunotherapy • randomized
	DTIC + placebo		PR: 12% DCR: 56% (no CR in either arm)	11.7 weeks	20%	51.3 weeks	• double-blind • placebo-controlled • no active brain metastases
Amaravadi et al. *(16)*	A: S + TMZ ex, –brain mets, –prior TMZ B: S + TMZ st, –brain mets, –prior TMZ C: S + TMZ ex, +/–brain mets, –prior TMZ D: S + TMZ st, + brain mets, + prior TMZ	II	PR: 24%; SD: 42% PR: 15%; SD: 58% PR: 0%; SD: 28% PR: 16%; SD: 50% (no CR in either arm)	5.9 months 4.2 months 2.2 months 3.9 months	50% 40% 11% 26%	–	• any number of prior therapies • +/– brain metastases • +/– prior TMZ
Agarwala et al. (PRISM Study) *(18)*	C/P + sorafenib	III	PR: 12% DCR: 66%	17.4 weeks	32%	42 weeks	• progressed on DTIC/TMZ • randomized • double-blind
	C/P + placebo		PR: 11% DCR: 62% (no CR in either arm)	17.9 weeks	29%	42 weeks	• placebo-controlled • no active brain metastases

BRR = best response rate, PFS = progression-free survival, OS = overall survival time, CR = complete response, PR = partial response, SD = stable disease, DCR (CR + PR + SD) = disease control rate; DTIC = dacarbazine, TMZ ex/st = temozolomide extended/standard dosing, C/P = carboplatin/paclitaxel.

be well tolerated. The maximum tolerated dose (MTD) has not been reached in this phase I trial. Of interest, there were no drug–drug interactions and the most frequently reported drug-related adverse events were grade 2 or less in severity. Fatigue, diarrhea, nausea, alopecia, and hand–foot skin reactions were the most dominant side effects. One RCC patient achieved a partial response, seven RCC patients and one melanoma patient showed a stable disease. In a biomarker substudy, there were no significant changes in the absolute values of lymphocytes, levels of proangiogenic cytokines, or inhibition of phosphorylated extracellular signal-regulated kinases in T cells or natural killer cells with this treatment combination *(19)*.

Few months later, two phase II clinical trials on sorafenib plus interferon-α as first- or second-line treatment in advanced metastatic melanoma have been published. In one trial the response rate was 33% including 28% partial and 5% complete responses. The median duration of response was relatively long (12 months). The median PFS was 10 months. Atypical adverse events have been observed. Most of the adverse events were likely related to the interferon-α2b treatment. The authors concluded that only a larger, randomized trial would determine whether there is any advantage to this regimen compared to sorafenib alone in RCC patients *(20)*.

Another phase II trial using the same dosages in the first-line situation only showed 19% objective confirmed responses and an additional 50% of patients with either an unconfirmed partial response or stable disease as best response. The median PFS was 7 months. However, in this clinical trial a pronounced toxicity that was greater than expected with either interferon or sorafenib alone was observed. The authors concluded that the toxicity of this combination limits the further development of sorafenib and interferon-α in combination *(21)*.

9. SORAFENIB AND PEGYLATED INTERFERON-α2B IN METASTATIC MELANOMA

Very recently, pegylated interferon-α2b has been demonstrated to improve the relapse-free and distant metastasis-free survival in patients with micrometastasis in the lymph nodes (stage IIIA, AJCC classification) in a large-sized EORTC trial on 1,254 patients in the adjuvant setting compared to observation alone *(22)*. The Dermatologic Cooperative Oncology Group (DeCOG) initiated a phase II trial in 10 centers out of Germany in November 2007. In this investigator-initiated trial, sorafenib is combined with pegylated interferon-α2b to improve the monotherapy activities of both agents. Sorafenib is used at the conventional dose of 400 mg bidaily, pegylated interferon-α2b is given subcutaneously with a dose of 3 $\mu g/m^2$ body surface once a week for a total of 8 weeks. The primary endpoint of this phase II trial is the disease control rate (CR + PR + SD) at 8 weeks. A translational biomarker study from blood and tumor tissue of the patients will be performed to correlate or ideally predict the treatment outcome with the implementation of biomarkers.

10. CONCLUSIONS

Looking ahead, the crucial question remains whether sorafenib as a single-agent is strong enough to inhibit mutated B-Raf. It is mainly unclear to date whether the inhibition of specific molecules in the signal transduction pathway alone is sufficient or whether the antiangiogenic properties of sorafenib are more crucial in inhibiting the tumor growth of melanoma cells. Today it appears that there is limited if any activity of sorafenib as a single agent in metastatic

melanoma. Also, the add-on of sorafenib to potent chemotherapy regimens like carboplatin/paclitaxel was not leading to an enhanced clinical activity. The results of randomized phase II trials on the combination of sorafenib with either DTIC or temozolomide showed encouraging results, but need to be confirmed in large-sized phase III trials before final conclusions could be drawn table 2. Without evaluating the therapeutic potential of sorafenib in new biomarker studies, the role of sorafenib in metastatic melanoma remains relatively unclear. The combination of sorafenib with other agents apart from signal transduction inhibitors in carefully conducted clinical trials will be crucial.

To give patients the best treatment, it is essential to conduct further clinical trials. The current standards of care such as DTIC have never been shown to effect the overall survival time or cure rate in metastatic melanoma patients. Therefore, physicians should preferentially refer patients with this "tough-to-treat-disease" to controlled clinical trials in specialized melanoma centers.

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48 Targeting Immunological Synapse: New Horizons in Immunotherapy for Cancer

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CONTENTS

ABSTRACT

Immunotherapy has been a part of armamentarium against neoplastic diseases for decades. Various approaches have been used with a very limited success. The site of the contact between a T cell and an antigen-presenting cell (APC) is named the immunological synapse, and it is characterized by an organized spatial redistribution of TCR–pMHC complexes and accessory molecules. For the last 10 years, several accessory molecules have been discovered and their function in T-cell stimulation has been described. The list includes co-stimulatory molecules (CD28, CD27, OX-40, 4-1BB, ICOS, CD40L) and inhibitory molecules (CTLA-4, PD-1, PD-2, BTLA). This knowledge led to the development of a new area in the immunotherapy for cancer—targeting the immunological synapse. Monoclonal antibodies blocking CTLA-4 are in advanced clinical testing; and 6–21% response rates have been observed in patients with metastatic melanoma with few relapses. Treatment with CTLA-4-blocking antibodies demonstrated the ability to break tolerance to self antigens, manifested by the development of side effects in the form of autoimmune diseases. Agonistic antibodies against CD40 have undergone phase I testing, and they showed activity in non-Hodgkin lymphoma and melanoma. Antibodies against 4-1BB, PD-1, and OX-40 are at earlier stages of clinical testing. After these antibodies have been tested as monotherapy, combination of two or three agents targeting accessory molecules appears to be a direction for future development.

Key Words: Immunotherapy; immunological synapse; CTLA-4; autoimmunity; melanoma; cancer

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The concept of using immunotherapy for treatment of patients with cancer is not new. Already Paul Ehrlich believed that it would be possible to induce powerful immune responses to eliminate cancerous foci as it is possible in the case of infection *(1)*. For the last hundred years thousands of experiments have been done on animals and a wealth of knowledge on tumor biology and the immune system has been gained, but the clinical realization of the proposed methods of immunotherapy has not lived up to its expectations yet. The myriad various approaches have been tested. The list, just to mention a few, includes non-specific stimulation of the immune system with bacterial antigens (BCG) or with cytokines (interferon, interleukin-2, GM-CSF, interleukin-12); more specific stimulation with a variety of vaccines (peptide, protein, tumor lysate, whole cell, heat shock protein, dendritic cell vaccines) or virally delivered antigens; activation of the innate immune responses (stimulation through toll-like receptors) and finally adoptive cytotoxic T-lymphocyte therapy *(2–5)*. Unfortunately, the response rates to immunotherapy range from 2 to 15%, and they are lower than response rates to traditional chemotherapy in the case of the majority of metastatic cancers *(5,6)*. Despite these somewhat disappointing results, there is a lot of enthusiasm among physicians using immunotherapy for cancer—although a few responses are seen, these responses are frequently durable, and patients can be cured of cancer *(2)*. Recent developments in the field of medical oncology, including the introduction of monoclonal antibodies and small molecules to the clinical practice, have led to the development of a new area of immunotherapy for cancer in which attempts are made to target the immunological synapse.

1. IMMUNOLOGICAL SYNAPSE

In order to elicit an immune response, T cells must interact with antigen-presenting cells (APC), mainly dendritic cells, macrophages, or B cells. This activation consists of two steps. The process of the recognition of a particular antigen presented by either major histocompatibility complex (MHC) class I or class II molecule by a T-cell receptor is antigen specific. The second step is antigen non-specific; it is an interaction between the co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2) on the surface of APCs and the activating ligand CD28 expressed by T cells.

The place of contact between a T cell and an APC is not just a space between two cell membranes armed with multiple interacting molecules. It has a complicated structure named the immunological synapse. This formation consists of a central supramolecular activation cluster (SMAC) of T-cell receptor (TCR)–MHC complexes together with engaged co-stimulatory molecules (CD28-B7 complexes) and a peripheral SMAC containing other accessory molecules (CD54-LFA-1). Bulky molecules (CD43, CD45) that would interfere with TCR–MHC interactions because of their large size are excluded from the site of contact *(7–10)*. The formation of this unique juncture is a dynamic process starting from initial interaction of integrins, followed by antigen-specific interaction between a TCR and an MHC–antigenic peptide complex leading to spatial redistribution of molecules on the surface of the cell membrane *(7)*. This process might be abrogated in the presence of antagonistic antigens *(11)*, and it is dependent on the biophysical processes within the membrane (lipid rafts) and changes of cytoskeleton in the cytosol *(10)*. It is known that additional co-stimulatory or inhibitory molecules are also present in the immunological synapse, although not all of them participate in synapse formation (Fig. 1) *(12)*. CTLA-4, the main inhibitory molecule, redistributes to the synapse only during the late stages of T-cell stimulation *(12,13)*. CD40 does not accumulate at sites of its ligand unless the TCR has been engaged *(12)*. CD70 is compartmentalized to the same vesicles as MHC class II, and they are routed together to the cell surface upon stimulation *(14)*. Ligands for other costimulatory molecules like OX40L and 4-1BBL are located in lipid rafts separate from the central rafts that contain MHCs *(15)*.

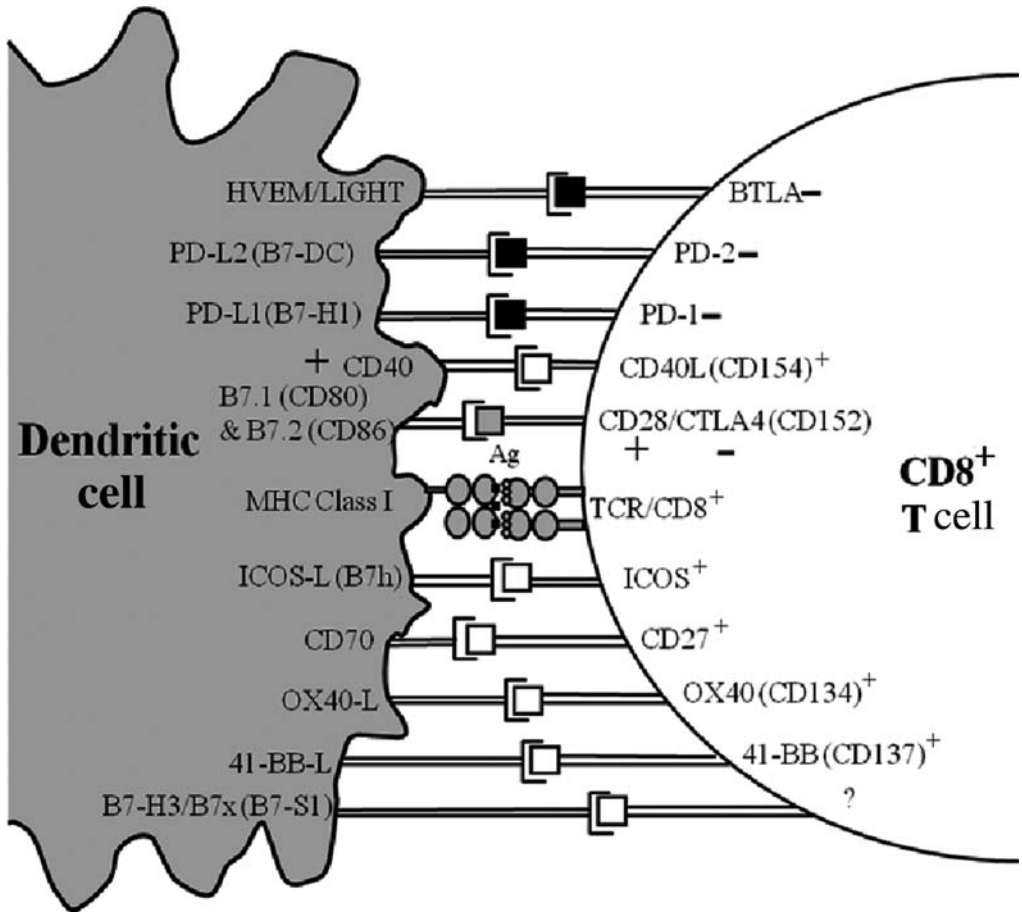

Fig. 1. Immunological Synapse—schematic depiction of the site of contact between a T cell and a dendritic cell with inclusion of the TCR–pMHC complex and accessory molecules. Black boxes show inhibitory molecules and white boxes show co-stimulatory molecules.

It is well accepted that accessory molecules play a pivotal role in modulation of T-cell responses. Therefore, it was a natural consequence that scientists attempted either to block inhibitory molecules or to activate stimulatory molecules in order to enhance immune responses, including antitumor responses. The opposite approach has been utilized in the treatment of autoimmune disorders *(16)*.

2. TARGETING INHIBITORY MOLECULES

2.1. CTLA-4 (CD152)

CTLA-4 is a co-inhibitory molecule present on the surface of activated, but not naïve, T cells. This molecule as well as CD28 belongs to the immunoglobulin family. Both molecules interact with CD80 (B7-1) and CD86 (B7-2) and cause opposite effects on T cells. The engagement of CD28 is required for T-cell activation; the engagement of CTLA-4 results in inhibition of immune responses *(17)*. These two molecules have also a similar effect on dendritic cells. Engagement of B7 molecules by CD28 results in activated DCs, whereas engagement of B7 molecules by CTLA-4 results in tolerogenic DCs *(18)*. CTLA4 is

characterized by an approximately 20 times higher affinity for CD80 and CD86 than CD28. Although both molecules exist as dimers, CTLA-4 can bind two B7 molecules whereas CD28 binds only one, thereby increasing the ability of CTLA-4 to compete with CD28 *(19–21)*. CTLA-4 inhibits immune responses not only through outcompeting CD28 in number and affinity, but also through causing variations in the organization of the immunological synapse *(13)* and through the recruitment of serine/threonine phosphatases *(22,23)*. The signaling through the TCR leads to activation of ZAP-70 and Lck kinases and subsequent phosphorylation of CTLA-4 and stabilization of its surface expression *(21,24)*. Recently it has been shown that CTLA4 has ability to increase T cell mobility and shorten the time of T cell/DC contact; this leads to a decrease in the level of T cell activation *(25)*. The importance of CTLA-4 as an immunoinhibitory molecule is emphasized by the phenotype of CTLA-4 knockout mice, which develop lethal lymphoproliferative disorders within weeks of birth *(26,27)*. It has been also postulated that CTLA-4 modulates immune responses by its presence in high levels on $CD4^+CD25^+foxp3^+$ regulatory T cells (Treg) *(28,29)*. It is also possible that CTLA-4 on the $CD4^+CD25^+foxp3^+$ regulatory T cells inhibits immune response by reverse signaling through B7 molecules on the surface of dendritic cells and induction of the immune suppressive enzyme indolamine 2,3 dioxygenase (IDO) *(30–32)*.

Several preclinical models revealed effectiveness of the CTLA-4 blockade with monoclonal antibodies in the therapy of cancer. In a murine model, systemic administration of the antagonistic antibodies to CTLA-4 elicited tumor regression for a variety of tumors *(33)* and decreased relapses when it was given as adjuvant immunotherapy in a model of metastatic prostate cancer *(34)*. When poorly immunogenic models were used, tumor rejection occurred only after prior vaccination with irradiated tumor cells secreting granulocyte-macrophage colony-stimulating factor (GM-CSF) *(35–38)* or after depletion of $CD25^+$ regulatory T cells *(39)*. Surprisingly, in the B16 melanoma model, treatment with anti-CTLA-4 antibody and GM-CSF-secreting tumor resulted in increased tumor infiltration not only by effector but also by regulatory T cells *(40)*, arguing that maybe not really the blockade of $CD4^+CD25^+$ regulatory T cells but rather the change of ratio between these cells and effector T cells is responsible for the mechanism of action *(41)*.

Two different fully human monoclonal antibodies against CTLA-4 have been developed for use in human studies: ipilimumab (formerly known as MDX-010) manufactured by Medarex, Inc., and Bristol-Myers Squibb, and tremelimumab (formerly known as CP-675,206 or ticilimumab) manufactured by Pfizer. Ipilimumab belongs to IgG1 and tremelimumab to IgG2 classes of monoclonal antibodies. Both antibodies were studied in dose-and-toxicity finding phase I and response-assessing phase II clinical trials. Currently they are undergoing phase III testing in patients with metastatic melanoma. Ipilimumab is being tested in a randomized clinical trial of dacarbazine versus the combination of dacarbazine and ipilimumab; tremelimumab—in a randomized clinical trial of dacarbazine or temozolomide versus tremelimumab. Phase I and phase II clinical trials involved mainly patients with malignant melanoma, but these antibodies have been also tested in patients with renal cell, prostate, lung, colon, and ovarian cancer *(42–49)*. Both ipilimumab and tremelimumab have been used as monotherapy, or combined with immunotherapy, or combined with standard therapy. As mentioned before, preclinical experiments revealed that immunotherapy may enhance antitumor effect of an antiCTLA-4 antibody against poorly immunogenic tumors. Therefore, attempts have been made to combine either of the antibodies with vaccines with dendritic cells pulsed with immunodominant melanoma-derived peptides, with oligodeoxynucleotide PF-3512676 (CPG 7909), with vaccines with irradiated GM-CSF-secreting tumor cells, or with treatment with high-dose interleukin-2 *(49,50)*. In clinical trials using standard therapy, ipilimumab or tremelimumab were combined with hormonal therapy in patients with prostate cancer and

breast cancer, with sunitinib in patients with renal cell carcinoma *(49)*, with dacarbazine in patients with melanoma *(51)*, and with docetaxel in patients with prostate cancer *(52)*. Overall, 6–21% of patients were noted to have either complete or partial responses. Interestingly, the majority of the responses are durable, and several patients remain free of the disease 5–6 years after initiation of therapy. These findings are consistent with responses seen in patients treated with high-dose interleukin-2, and they are so different from the more common short-term responses seen in patients treated with chemotherapy. The list of grade 3 and 4 toxicities included primary autoimmune disturbances (enterocolitis, hepatitis, hypophysitis, thyroiditis, dermatitis, nephritis, rheumatoid arthritis-like syndromes) in 15–43% of patients. The majority of these symptoms resolved without intervention or after treatment with steroids; in rare (less than 1%) cases colitis led to bowel perforation or megacolon requiring colectomy. It is important to note that the median time to the best response is prolonged (several months to years in one series); occasionally the responses were seen after a period of stabilization or even progression of the disease *(53)*. These observations make decisions on response assessment and therapy discontinuation quite challenging *(49,50)*.

2.2. Programmed Death-1

The programmed death-1 (PD-1; CD279) molecule is a transmembrane molecule present on activated T cells, B cells, and monocytes in high levels and on a minor population of $CD4^{-}CD8^{-}$ thymocytes *(54,55)*. Its extracellular domain shares 24% homology to CTLA-4 *(56)*. PD-1 binds to two different receptors PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273). PD-L1 is constitutively expressed by DCs, T cells, B cells, macrophages, mesenchymal stem cells, cultured mast cells and inducible on monocytes and keratynocytes. It is also expressed on a variety of nonhematopoietic cell types *(55,57)*. Cell activation results in the upregulation of the expression of PD-L1. Moreover, PD-L1 expression has been described on various human malignant cells, and it correlates with poor prognosis *(58,59)*. In contrary, PD-L2 is expressed constitutively on monocytes, and it is downregulated upon activation *(56)*.

As opposite to the rapid development of a lymphoproliferative disorder in CTLA-4 knock-out mice (mice die within 3–4 weeks of birth), PD-1 knock-out mice initially develop and grow apparently normally. They were characterized by increased cellularity of the peripheral lymphatic organs, increased level of IgG3, and the phenotypical alteration of the peritoneal B-cell population *(54)*. As these mice aged, they developed autoimmunity in the form of typical lupus-like glomerulonephritis and destructive arthritis *(60)*. When these mice were crossed on BALB/c background, the development of T-cell-mediated cardiomyopathy was observed *(61)*.

Experiments in which PD-1 was engaged by PD-L1-Ig or PD-L2-Ig fusion protein revealed that PD-1 transduces an inhibitory signal to T cells and B cells, and it leads to decreased cell proliferation and cytokine production *(62,63)*. Furthermore, expression of PD1 is increased in virus-specific T cells in the setting of chronic infections, suggesting that blockade of PD1 signaling may reverse a state of T-cell "exhaustion" *(64)*. Interestingly, some studies revealed that PD-1 ligands may also enhance T-cell activation, but it remains uncertain if these findings have a biologic significance or were just dependent on the experimental design *(65,66)*. It has been implicated that the PD-1–PD-L pathway has a distinct function in regulating peripheral tolerance and autoimmunity, and it is most effective in limiting responses in situations involving low-affinity TCRs or weakly activated T cells *(55,67)*. Since T cells with antitumor specificity may belong to this group of "exhausted" T cells, blocking the PD-1 signaling became an attractive target in immunotherapy of cancer, and phase I clinical trials with a fully human anti-PD-1 monoclonal antibody (MDX-1106/ONO-4538) has been initiated *(30)*.

3. TARGETING CO-STIMULATORY MOLECULES

3.1. CD28

As discussed in the section on the structure of immunological synapse, CD28 is a pivotal co-stimulatory molecule present on the surface of T cells, and it would seem to be an attractive target to enhance immune responses with a use of an agonistic antibody. A superagonist antibody against CD28 (antibody that activates T cells without crosslinking of a TCR)—TGN1412 has been tested in humans with deleterious consequences. It was manufactured by TeGenero and tested by a contract research organization Parexel. Six volunteers were simultaneously infused, and all six developed a cytokine storm and multiorgan failure *(16,68,69)*.

3.2. 4-1BB

4-1BB (CD137) is a co-stimulatory molecule that belongs to the tumor necrosis factor (TNF) receptor superfamily *(70)*. It was initially identified as an inducible co-stimulatory receptor on T cells *(71,72)*, but subsequently its expression was discovered on splenic, follicular and bone marrow-derived DCs, monocytes, activated NK cells, and activated eosinophils *(70)*. A single ligand, named 4-1BBL (CD137L), has been characterized, and it is expressed on activated macrophages, DCs, and B cells. CD40 plays a role of a major regulator of 4-1BBL expression *(73,74)*. Interestingly and surprisingly, the 4-1BBL is also expressed and functional on some carcinoma cells. Cancer cells that were stimulated via 4-1BBL produced interleukin-8, a potent chemoattractant for T cells, neutrophils, and basophils, what could be considered disadvantageous for tumor survival *(75)*. When 4-1BBL was introduced with a retrovirus into sarcomas or lymphomas, these tumors were not able to engraft in syngeneic mice because of strong antitumor T-cell responses *(76)*. 4-1BB signaling results in the activation of nuclear factor (NF)-κB pathway, upregulation of anti-apoptotic molecules bcl-X_L, bfl-1, protection from activation-induced T-cell death, increased T-cell survival, and it can partially substitute signaling via CD28 *(70,77,78)*. In humans, stimulation of CD8 T cells with 4-1BBL results in upregulation of effector molecules, like perforin and granzyme A, and in full differentiation of CD8 memory T cells *(70)*. While 4-1BBL, especially in vitro, can activate both CD4 and CD8 T cells, agonist antibody against 4-1BB effects CD8 T cells preferentially *(79–81)*. In addition, this antibody has the ability to reverse anergy induced by soluble antigens *(82)*.

In the preclinical models, an agonist antibody to 4-1BB has been shown to augment tumor-selective cytolytic T-cell activity against sarcomas, mastocytomas, gliomas markedly, and this process led to tumor eradication *(74,83,84)*. In cases of poorly immunogenic tumors (TC-1 lung carcinoma, B16-F10 melanoma, JC breast cancer, MCA26 colon carcinoma) only was the treatment successful when it was combined with vaccine with tumor-antigens, interleukin-12 gene transfer, or intratumoral delivery of adenoviral-mediated gene transfer of the 4-1BBL *(85–88)*. In addition, the treatment with anti-4-1BB antibody enhanced antitumor activity of adoptively transferred T cells *(89)*. These encouraging results led to development of a humanized anti-4-1BB agonist monoclonal antibody (BMS-663513) that is currently being tested alone or in combination with chemotherapy or radiotherapy in early clinical trials in patients with advanced solid tumors.

3.3. OX-40

OX40 (CD134; TNR4) is a late co-stimulatory molecule belonging to TNF family that is present on the surface of activated, but not naïve, $CD4^+$ and $CD8^+$ T cells *(90,91)*. Its ligand, OX40L (GP34), has been identified on activated DCs, B cells, and macrophages *(70,91)*.

Ligation of OX40 serves as a stimulus for T cells for a prolonged proliferation, especially beyond day 4 after stimulation and it assures development of memory T cells *(91,92)*. Since agonistic antibodies to OX40 and OX40L:Ig were shown to enhance antitumor responses in several animal models *(93–95)*, OX40 has become the next attractive target for the immunotherapy of cancer. Currently an agonistic murine antibody to OX40 for use in humans is in the early stages of development *(30)*.

4. TARGETING APCS

4.1. CD40

CD40 is another member of the TNF superfamily, and it is expressed on B cells, DCs, macrophages, activated T cells, some endothelial and epithelial cells and on a broad range of hematological and epithelial malignancies *(96,97)*. Interactions between CD40 and its ligand CD40L (CD154) play a pivotal role in governing humoral and cell-mediated immunity. Engagement of CD40 leads to B-cell clonal expansion, germinal center formation, isotype switching, affinity maturation, and generation of long-lived plasma cells *(96)*. Engagement of CD40 on dendritic cells has been shown to lead to dendritic cell maturation and to induction of production and secretion of IL-12 *(98)*. In addition, several models demonstrated that engagement of CD40 receptor on DC could bypass the need for help from $CD4^+$ T cells, and it resulted in the potent activation of $CD8^+$ CTLs *(99–101)*. Finally, CD40 ligation on macrophages induces production of reactive oxygen species, matrix metalloproteinase activity, and production of proinflammatory cytokines *(102,103)*.

Blocking CD40–CD154 interactions may be either harmful or beneficial depending on a disease model *(104)*. Individuals carrying mutations in the *CD154* gene that render the protein nonfunctional develop hyper-IgM syndrome (HIM), a immunodeficient state in which, although patients have a normal number of T and B cells, they produce mainly IgM antibodies and fail to switch isotypes in response to T-cell-dependent antigens *(105)*. Animal experiments revealed that disrupting CD40–CD154 interactions leads to increased susceptibility to infection with *Leishmania major* and *Pneumocystis carinii(106,107)* and renders tumor vaccines ineffective *(108)*. At the same time blocking CD40–CD154 interactions may be helpful in treating autoimmune diseases in mice, in particular, collagen-induced arthritis, lupus nephritis, autoimmune thyroiditis, diabetes, experimental autoimmune encephalitis, and inflammatory bowel disease *(104)*. Moreover, treatment with a blocking antibody against CD154 reduced the size of aortic atherosclerotic lesions in hyperlipidemic mice *(109)*.

Using CD40 as a target in immunotherapy for cancer is not as self-explanatory as in the cases of the aforementioned molecules. CD40 is expressed not only on APCs which stimulate T cells to antitumor activity, but its expression has also been demonstrated on several hematologic (leukemia, lymphoma, myeloma) and nonhematologic malignancies (breast, lung, ovary, bladder carcinoma, melanoma, sarcoma) *(97)*. Interestingly, while ligation of CD40 delivers a proliferation and survival signal to normal B cells, and this phenomenon can be also observed in malignant B cells, stimulation via CD40 may induce apoptosis in breast, ovarian cancer, or melanoma cells *(110–112)*. In addition, monoclonal antibodies specific for CD40 can exert antitumor effect on $CD40^+$ malignancies by mediating antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) *(30,112)*. Therefore, by using agonist monoclonal antibodies or soluble recombinant CD40L in patients with cancer, it is possible to obtain a simultaneous benefit through stimulating immune system and through the direct effect on a tumor.

Several phase I clinical trials targeting CD40 have been performed. In one trial, a recombinant CD40L was used in 32 patients with advanced solid malignancies, and two partial responses were

seen. The therapy was complicated by transient elevation of liver transaminases *(113)*. Moreover, two agonist antibodies, CP-870,893 and SGN-40, have been tested in two separate phase I clinical trials in patients with advanced solid malignancies and non-Hodgkin's lymphoma, respectively. Partial responses were seen in about 15% of patients. Grade 1-to-2 cytokine release syndrome was observed, and it presented with chills, rigors, fever, nausea, vomiting, muscle aches and back pain, and elevation of serum Il-6 and TNFα levels minutes to hours after infusion *(114,115)*. Only few of the partial responders after a longer follow up achieved complete responses. These observations make the pattern of responses quite different from the one seen in patients treated with anti-CTLA4 antibodies, and it possibly suggests that antitumor activity comes rather from a direct effect of the anti-CD40 antibody on cancer than stimulation of the immune system *(112)*. Further studies with these agents alone or in combination therapy are ongoing.

5. AUTOIMMUNITY

It appears intuitional that immunotherapy for cancer should be closely related to autoimmunity. Cancer arises from autologous tissue, and autoimmune diseases are characterized by immune responses against self-antigens, so successful immunotherapy targeting cancer may potentially target host's healthy organs. Humans use multiple control mechanisms that assure that autoreactive cells either do not develop or do not proliferate uncontrollably. These processes are generally grouped as central and peripheral tolerance. Central tolerance describes mainly the thymic process of negative selection in which T cells carrying a T-cell receptor with high avidity for self-antigens are deleted. It is well known that some of T cells that are able to recognize self-antigens escape from negative selection, but in the peripheral organs they become anergic after they interact with tissue-specific antigens presented in the absence of co-stimulatory signals. Other control mechanisms include immunological ignorance (T cells and B cells coexist with antigens, but they are not stimulated); the presence of cryptic antigens (immune cells are not able to get into the compartments where antigens are present); the presence of antigens in immunologically privileged sites; upregulation of surface expression of inhibitory molecules or direct inhibition by interacting cells. Tumors escape rejection by the immune system by using similar mechanisms: activating antigens are not available for the immune cells; co-stimulatory and adhesion molecules are downregulated and inhibitory molecules are upregulated on the tumor surface; MHC molecules are downregulated; immunosuppressive cytokine are produced by tumor *(116)*. In order to generate successful immunotherapy these control mechanism must be overcome, and therefore the therapy can possibly lead to autoimmune side effects *(6)*.

Development or exacerbation of autoimmune disease (e.g., thyroiditis, thrombocytopenia, rheumatoid arthritis, systemic lupus erythematosus, sarcoidosis, psoriasis) with symptoms ranging from mild to life-threatening or fatal has been observed in patients receiving interferon alpha-2b *(117–124)*. It has been noted that 20–30% of melanoma patients treated with interferon alpha-2b in the adjuvant setting develop autoimmunity in the form of the overt disease or in the form of the induction of autoimmune antibodies *(125,126)*. Recent studies, both prospective and retrospective, revealed that the appearance of autoantibodies or clinical manifestations of autoimmunity was associated with statistically significant improvements in relapse-free survival and overall survival in melanoma patients *(125,127)*. One study reported a dramatic influence of autoimmunity on cancer, and it showed that melanoma recurred only in 13% of patients with signs of autoimmunity and in 73% without autoimmunity *(125)*. Similar findings describing the correlation between autoimmunity and the outcome were seen in patients treated with high-dose interleukin-2 *(118–121,128–130)*. Autoimmune disorders

were also the main adverse effects of treatment with tremelimumab and ipilimumab, two anti-CTLA-4 antibodies, although the correlation between the autoimmunity and anticancer activity is less clear *(49,50,125)*. Interestingly, the list of autoimmune disorders seen in patients treated with anti-CTLA4 antibodies includes entities that are observed in patients treated with interferon alpha or interleukin-2-like thyroiditis, vitiligo, autoimmune hepatitis, rheumatoid arthritis-like syndrome, but it also includes a different set of complications, like colitis, hypophysitis, and dermatitis. It suggests that there are different mechanisms behind the efficacy of these two groups of medications. Moreover, it demonstrates how limited our knowledge is on the regulation of the immune responses to self despite thousands of pre-clinical experiments.

Despite the aforementioned data one could still argue these findings are coincidental. It cannot be excluded that patients who developed autoimmunity had a better prognosis a priori even in the absence of the therapy. This bias could influence retrospective studies quite strongly, but it should not have such a significant impact on prospective studies. In addition, the presence of a lead-time bias cannot be completely excluded, since signs or symptoms of autoimmunity do not always precede the recurrence or progression of the disease. When ipilimumab was used in the adjuvant setting, for many individuals, it took several months before they developed autoimmunity *(131)*. Next, autoimmunity is an immune response against an antigen within a body of a host. This definition does not distinguish whether the response is innate or acquired, whether it is mediated by antibodies or cellular mechanisms. It was postulated that the mechanisms of action of interferon included upregulation of MHC molecules, stimulation of dendritic-cell maturation, enhancement of the function of effector cells, antiangiogenic effects, and antiproliferative effects on tumor *(125,126)*; anti-CTLA4 antibodies work on the immunological synapse, therefore the blockade will enhance T-cell function directly and possibly B-cell function indirectly via $CD4^+$ cells. It is naive to believe that the measurement of a limited number of autoantibodies or registration of the development of an autoimmune syndrome would reflect the whole activation of the immune system correctly. It leads to a compelling postulate that not only should a better-defined set of tests and syndromes but also the well-defined cutoff intensity be used in clinical trials addressing the relation between autoimmunity and anticancer responses. The data on autoimmunity and immunotherapy for cancer are even more complicated by the fact that autoantibodies can be detected in a significant number of healthy individuals. Antinuclear antibodies (ANA) are detected in 32 and 13% of healthy individuals at the 1:40 and 1:80 dilutions, respectively *(132)*. Finally, probably not every sign or symptom of autoimmunity is equally important, it is reasonable to believe that development of vitiligo is more important for patients with melanoma than prostate cancer *(133)*. Since more and more patients are treated with anti-CTLA4 antibodies and the side effects they develop are studied more closely, since the data from the adjuvant interferon studies are reanalyzed carefully in regard of the autoimmune phenomena, since new therapeutic agents against immunological synapse are in development, hopefully we will be able to clarify the relation between antitumor response and autoimmunity in the near future.

6. WHERE DO WE COME FROM? WHAT ARE WE? WHERE ARE WE GOING?

Development of cancer immunotherapy has taken a long and winding road since the observation by Paul Ehrlich that stimulation of the immune system can lead to tumor rejection *(1)*. Although only a limited number of individuals respond to this method of therapy, it continues to be very appealing for oncologists and their patients. Recent enormous progress in medical oncology translates mostly into a prolonged survival or improved quality of life of patients

with metastatic cancer, but hardly ever gives a chance for cure. This leads to an argument that a 10% response rate to dacarbazine and a 10% response rate to tremelimumab or ipilimumab in patients with metastatic melanoma do not have the same meaning. We know that virtually all responders to chemotherapy progress eventually, while the majority of responders to CTLA-4-blocking antibodies are able to get rid of the disease. These findings also suggest that an overall survival and not a response rate should be the end points of randomized clinical trials with immunotherapy, and that despite our eagerness to see data, the trials should have a prolonged follow-up and not be analyzed prematurely.

There is another reason that targeting of the immunological synapse, which is a new field in tumor immunotherapy, leads to a growing enthusiasm. Monoclonal antibodies or small molecules, in opposite to dendritic cell vaccines or adoptive transfer cell therapy which require specialized facilities and are prepared only in a limited number of centers, can be manufactured by pharmaceutical companies for use in general oncologic practice. Moreover, they do not require sophisticated methods of administration, and the treatment is usually well tolerated by patients. At the same time clinicians must become familiar with a new group of side effects that are not seen in patients treated with traditional chemotherapy. Treatment with CTLA-4-blocking antibodies can be complicated by development of autoimmune disorders which are self-limiting or mild in most of the cases, but they can occasionally be very severe, leading to long-term disability *(49,50)*. As we gain more experience with the use of these biologicals, algorithms for the approach to the management of their side effects are developed and hopefully will result in the decrease of long-term sequelae *(50,134)*. We could assume that treatment with antibodies targeting 4-1BB, PD-1, or OX-40 will result in similar complications, but the complete spectrum cannot be predicted. When an agonistic antibody against CD40 was used, patients did not develop autoimmune phenomena, but their treatment was complicated by the cytokine release syndrome *(112)*. In addition, the dramatic adverse effects noted in the CD28 superagonistic antibody clinical trial *(16,68,135)* showed that we must not forget that introduction of new treatments is pursued under constant scientific and ethical scrutiny, so the risks of harm to patients are diminished as much as possible.

Despite the exciting results coming from targeting the immunological synapse, it is too early to fete on the success of the immunotherapy for cancer. Still very few patients respond to the therapy, side effects might occasionally be quite severe and we are currently not able to identify potential responders before the initiation of the therapy. Promising results originate from the preclinical experiments on combination of immunostimulants. Combining anti-CTLA-4 antibody with an anti-4-1BB antibody resulted in increased antitumor activity and decreased severity of autoimmune side effects *(136)*. Increased antitumor responses were also seen when anti-OX40 and anti-4-1BB antibodies or anti-B7-H1 and anti-PD-1 antibodies were used together *(137,138)*. Gene transfer of 4-1BBL and soluble PD-1 into murine hepatocellular carcinoma also triggered enhanced antitumor immunity *(139)*. Finally, a dramatic effect was seen when a combination of anti-CD40, anti-4-1BB, and anti-DR5 (death receptor 5) antibodies was administered to mice with metastatic fibrosarcoma *(140)*. Combination treatments open new horizons in targeting immunological synapse, but we must remember that these treatments may come with new toxicities; therefore, all clinical trials should be planned carefully and conducted thoroughly.

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49 Cutaneous Metastases of Melanoma: New Treatment Options

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CONTENTS

ABSTRACT

Cutaneous metastases are particularly frequent in melanoma of the skin, and they may occur as locoregional satellite or intransit metastases or likewise as distant cutaneous metastases. In satellite and intransit metastases, achievement of complete tumor remission may result in permanent cure. Therefore, the complete tumor remission of multiple cutaneous metastases is an important therapeutic goal in a subgroup of melanoma patients.

In small cutaneous metastases, the most effective approach is intratumoral treatment with interleukin-2 (IL-2), which results in complete remissions in 80–90% of tumors treated. Low dosages of 0.1–1 MIU IL-2 per tumor and 5–10 applications are sufficient to achieve complete remissions. Alternative immunological treatments are topical application of the toll-like receptor agonist imiquimod and the obligatory contact allergen dinitrochlorobenzene (DNCB), which likewise result in partial to complete remissions. The latter substance has also been combined with dacarbazine. Large cutaneous and soft tissue metastases are suitable for intratumoral chemotherapy in combination with electroporation. Bleomycine is an effective drug for such intratumoral treatments. Even large and bulky tumors may undergo dramatic regression and subsequently become operable.

In conclusion, several effective local treatment approaches have been developed for cutaneous melanoma metastases which may be preferred to systemic drug treatments in certain melanoma patients.

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1. INTRODUCTION

Cutaneous metastases are frequent events during tumor progression in cutaneous melanoma. Most frequent are satellite and intransit metastases as manifestations of regional metastases of melanoma *(1,2)*. As long as only single or few cutaneous metastases develop, surgical treatment is the option of choice. However, frequently multiple metastases develop simultaneously and they are recognizable already in a very early stage. At time of diagnosis, these cutaneous metastases often measure only few millimeters in diameter. It is not an uncommon situation that 50 or 100 or even more of these metastases develop simultaneously. Under these circumstances, surgical treatment is no longer an option and also radiotherapy of larger areas involved by cutaneous metastases is not indicated. Systemic chemotherapy has low response rates in metastatic melanoma and should be preferentially administered in the situation of distant metastases *(3)*. Therefore, effective topical treatments applied intratumorally or topically at the skin surface would be a desired option in these cases.

In the frame of distant metastasis, cutaneous metastases develop likewise frequently. Similarly as in intransit metastases, in distant metastases multiple lesions can develop simultaneously. Cutaneous metastases can be particularly harmful to the patients in a psychological view, because they are visible and a stigma for the patient. If systemic treatment achieves no response of the cutaneous metastases, topical treatments may likewise be a good treatment alternative.

Mainly three local treatment options with different drugs have been described so far in the literature. Dinitrochlorobenzene (DNCB) is an obligatory contact allergen and induces a pronounced inflammatory reaction. It is applied topically on the metastases and treatment is most of*ten* combined with systemical darcarbazine *(4)*. Interleukin-2 (IL-2) is an immune-stimulatory cytokine which has been used in the systemic treatment of distant melanoma metastases at maximum tolerated dosages *(5)*. Applied in an intralesional fashion, low dosages of IL-2 proved to be highly effective *(6)*. Imiquimod is a drug binding to toll-like receptor 7, which stimulates the response of the natural immune system. An inflammatory reaction results and cancer cells are rejected. Approved for the treatment of actinic keratosis and superficial basal cell carcinoma, Imiquimod has been shown to be likewise effective in Lentigo maligna and in superficial melanoma metastases *(7)*.

2. TOPICAL TREATMENT WITH 2–4 DNCB

The possibility of topical treatment with 2–4 DNCB has been first described by Malek-Mansour in 1973 *(8)*. DNCB is an obligate contact allergene and induces a pronounced inflammatory reaction. The Dutch oncologist Rumke was the first to combine DNCB treatment with systemic dacarbazine *(9)*. Increased response rates in comparison to dacarbazine alone have been reported. However, only small, non-randomized monocentric trials have been performed. Some reports suggested that the combined treatment schedule with DNCB and dacarbazine may improve the therapeutic efficacy of dacarbazine in the treatment of metastatic melanoma. Similar observations were reported from a mouse model *(10)*.

The treatment starts with sensitization using 2% DNCB in vaseline which is applied directly on the skin overlying the metastases and left there for 2 days. Thereafter, all skin metastases are treated

weekly with the concentration of DNCB ranging from 0.005 to 2%, eliciting a strong contact dermatitis. After 3 weeks, 48 hours subsequent to the last epifocal DNCB-application, dacarbazine is administered i.v. in a single dose of 850 mg/m^2. Cycles are repeated every 3 weeks *(4)*.

The largest trial reported is a retrospective survey with nine German centers, which evaluated 72 patients treated from 1993 to 2005 *(4)*. In stage III melanoma patients with intransit and satellite metastases ($n = 39$), the objective response rate was 62% [complete responses (CR) 39%]. In stage IV melanoma ($n = 33$) the objective response rate was only 9% (CR 3%). Thus, the analysis of the trial patients showed that responses were rare in distant metastases, but more frequent and long lasting in loco-regional disease. This clear-cut difference in response to treatment between patients with loco-regional and with distant skin metastases of melanoma is not surprising, but it was not described in previous reports in which patients with different disease stages were mixed. Therefore, the former impression that the combination of dacarbazine with topical DNCB treatment is more effective than dacarbazine alone is not supported by these data. It remains unclear if there is any synergistic action of dacarbazine and DNCB or if the same results would also be achieved with DNCB alone.

3. INTRATUMORAL IL-2 TREATMENT

IL-2 has been proven to be active in metastatic melanoma. Treatment of patients with metastatic melanoma with high-dose IL-2 resulted in a response rate of 20%. Fifteen percent of patients with high-dose IL-2 treatment developed partial responses and another 5% developed complete responses *(11–13)*. It is known that a certain percentage of patients with objective responses to IL-2 develop durable complete responses and may be regarded as cured *(14–16)*. This is the reason why the US Food and Drug Administration approved high-dose IL-2 treatment for patients with metastatic melanoma.

IL-2 has been likewise used in low-dose regiments, either alone or in combination with interferon-α and chemotherapeutic drugs. There are a number of comparative trials which show that the addition of low-dose interferon-α to chemotherapy or bio-chemotherapy does not increase the response rates or prolong the overall survival *(3,17–21)*. These data are in favour of the hypothesis that low-dose IL-2 treatment is not effective in metastatic melanoma.

Highest dosages of IL-2 in the tumour tissue can be achieved by intratumoral injection of IL-2. This is the rationale for the intratumoral treatment approach. The disadvantage of intratumoral application is clearly that only a limited number of tumour manifestations can be reached by this method. The advantage of intralesional IL-2 therapy is that it is only associated with low toxicity. A phase II trial in 24 patients with the intralesional IL-2 therapy did not record grade 3 and grade 4 toxicity except of one case with grade 3 headache *(6)*.

3.1. Indication for intratumoral IL-2 treatment

Intratumoral application of IL-2 can be easily administered in skin metastases and soft tissue metastases close to the skin. This is the main indication for intratumoral IL-2 application. If there is only a single or few metastases, surgical excision should be the treatment of choice. However, if there are multiple satellite or intransit metastases, intralesional IL-2 treatment with a high local response rate is presently the superior treatment option. This is particularly true as long as metastases are confined to the regional lymphatic drainage area.

In a high percentage of patients with multiple skin and lymph node metastases in stage III disease the metastatic spread can be controlled by intralesional IL-2 injections *(6)*. This is particularly the case if the start of treatment is early and the metastatic lesions are still small in diameter. Metastases up to a diameter of 5 mm respond as a rule very rapidly to intratumoral IL-2 injections. Therefore,

this treatment option is also effective if it is started in multiple metastases in the early phase of their development. Treatment of 50 or 100 lesions subsequently or simultaneously can still produce complete responses of all metastases treated. The disease control obtained by this treatment strategy may be probably associated with prolongation of survival in a certain percentage of patients.

The indication for treatment of distance skin metastasis may be regarded as doubtful because in the large majority of cases simultaneously metastases in visceral organs are present. Therefore, the treatment of the cutaneous metastasis may not impact the course of the disease. However, treatment of visible skin metastasis is appreciated by the patients, particularly if it is associated with regression and response of the treated lesions. Therefore, this treatment option may be considered in patients who have mainly skin metastases or have only limited visceral metastatic involvement.

3.2. Management of intratumoral IL-2 treatment

For intratumoral IL-2 treatment a stock solution is prepared containing 18 MIU recombinant human IL-2 (Proleukin) in 6 ml aqueous solution containing 5% glucose and 1% human albumin *(6)*. If the solution is prepared with purified water only, the injections are much more painful. The low amount of aqueous solution has the advantage that only small amounts of fluid have to be administered to metastatic lesions.

In order to limit toxicity, the first treatment session should be started with 1.5–3.0 MIU IL-2. The treatment should be started with the largest metastasis and subsequently additional metastases should be injected in further sessions. The total dose per treatment session should not exceed 12 MIU, in order to limit systemic toxicity. Up to 20 small cutaneous lesions have been treated simultaneously. As a rule, we performed three treatment sessions per week for 2–4 weeks. The recommended dosages per single lesion and the recommended duration of treatments dependent on the size of the lesion are given in Table 1.

3.3. Tumor responses under intralesional IL-2 therapy

A few cases with cutaneous metastases and complete regression after intralesional administration of IL-2 have first been described by Gutwald et al. (1994) *(22,23)*. We treated the first case in our institution in 1998, and after observing the complete regression of several cutaneous metastases, we started a phase 2 trial with IL-2 in soft tissue melanoma metastasis *(6)*. In this monocenter trial, a total of 24 patients (16 with stage III disease, 8 with stage IV disease) have been treated with intralesional IL-2. A complete response of the treated metastases was achieved in 15 patients (62.5%), in 5 patients partial responses were achieved, and in another 3 patients progressive disease was observed. The therapy was generally well tolerated. The observed adverse events were nearly exclusively of grade 1–2 toxicity. In these 24 patients 245 metastases were treated with complete response in 209 (85%) and partial response in 21 (6%). All together,

Table 1
Treatment Regimen

Lesion size: maximal diameter (mm)	*Single-dose MIU*	*Stock solution (mL)*	*Duration of treatment (weeks)*
<5	0.6	0.2	2
>5	1.2	0.4	3
>10	3.0	1.0	4
>15	6.0	2.0	4

objective responses have been observed in more than 90% of the metastasis treated. Subsequently, a number of additional cases successfully treated with intralesional IL-2 have been reported from other departments of dermatology *(24,25)*.

Since 2003 we started a second phase II trial and so far 25 patients are evaluable (data unpublished). More strict criteria for the inclusion of patients were required in this trial and 22 of 25 patients treated presented with stage III disease. In these patients we obtained objective responses in all patients treated. In 17 of 25 patients (68%) complete remissions of all metastases treated were achieved. Thus, the results of the first phase 2 study are confirmed and a nearly identical complete response rate of more than 60% of the patients was achieved.

No systemic responses have been observed after intralesional IL-2 treatment. The response is obviously confined to the local tumor. Therefore, injections of numerous tumors are required if multiple metastases are present. Best responses are seen in small tumors. Large bulky tumors which exceed a diameter of about 2 cm do most times not respond to the IL-2 treatment.

The only valid method of response assessment is to perform a biopsy and a histopathologic evaluation. As intralesional IL-2 treatment produces an inflammation, the nodules may even seem to be enlarged after the start of the treatment. The inflammatory response cannot be distinguished from the tumor tissue by ultrasound examination. Therefore, there is no secure response evaluation by use of sonography. The regression of the metastasis after IL-2 treatment is slow and may take months until the lesions completely disappear. If several lesions are treated simultaneously, we recommend taking a single biopsy 4–6 weeks after start of treatment in order to confirm the regression of the metastasis.

4. TOPICAL TREATMENT WITH IMIQUIMOD

Imiquimod is a toll-like receptor-7-agonist which enhances the innate immune response, enhances dendritic cells survival, and promotes tumor antigen-specific T-cell priming *(26)*. Imiquimod has been shown in larger phase III trials to be effective in genital wards, actinic keratosis, and superficial basal cell carcinoma and is approved for these treatments *(7,27)*. It has been used in the treatment of lentigo maligna, the in situ form of lentigo maligna melanoma. A number of case reports observed complete remissions of lentigo maligna under imiquimod treatment *(28–31)*. Several larger case series were reported in the literature. Powell et al. *(32)* treated 12 patients with facial lentigo maligna and 10 patients showed complete clearance which was histologically confirmed . Naylor et al. *(33)* reported a series of 28 evaluable patients with lentigo maligna from which 26 were complete responders . Follow-up of more than 1 year did not reveal any relapses.

Treatment with topical imiquimod was also tried in cutaneous intransit metastases. Bong et al. *(34)* treated 3 patients and achieved complete response in 2 of them by application of imiquimod under occlusive conditions during a period of 21–28 weeks . Only few more cases have been reported *(35,36)*. Others reported on combined treatments of imiquimod and intralesional IL-2 *(37)*. Together, 13 patients with 182 metastatic lesions were treated. The overall response rate was 55% and complete response rate was 47% in regard to the number of lesions treated. Thus, these response rates were inferior to those reported for consequent treatment with IL-2 alone.

5. DISCUSSION

Several drugs have been shown to be effective in the treatment of cutaneous melanoma metastases, particularly in intransit and satellite metastases in stage III. The first drug which was used for this kind of treatment was DNCB. The largest case series published by Terheyden et al. *(4)* showed a complete response rate in stage III melanoma of 39% . It remains unclear, if in this combined treatment schedule of systemic dacarbazine and topical DNCB the systemic dacarbazine treatment is necessary. It has been formerly described that DNCB alone is able to induce complete tumor remissions. However, DNCB as an obligate contact allergen causes severe contact dermatitis, itching, and crusting and thus lowers the quality of life of cancer patients. This trial disproves the impression that in stage IV disease the additional treatment with DNCB improves the efficacy of dacarbazine.

Most effective, particularly in stage III disease, seems to be the intralesional application of IL-2 *(6)*. More than 60% complete responses regarding the treatment of patients and more than 80% complete responses regarding the treatment of metastases were reported. Another advantage is the low-toxicity profile of this kind of treatment. Mild systemic IL-2 reactions with some fever and fatigue were observed. The local inflammatory response cannot significantly cut down the quality of life of the patients treated.

It is important to understand that long-term durable responses have been achieved by the intralesional IL-2 treatment *(3,6)*. The exact mechanism of the tumor response remains unknown. After an injection of IL-2 a dense inflammatory infiltrate around the tumor and infiltrating the tumor is observed. The tumor cells undergo apoptosis as we could show by immunohistochemical stainings that an increased expression of caspase-3 is observed *(6)*.

Imiquimod has so far been used only in few patients with cutaneous metastases. Probably, many more investigators tried imiquimod in cutaneous metastases than reports were published in the literature. In some reports, intralesional IL-2 has been additionally used. Another recent report first observed no response to topical imiquimod, but after additional application of tazarotene a response could be observed *(38)*. In contrast, the efficacy of imiquimiod seems to be clearly better in lentigo maligna lesions. These are confined to the epidermis and therefore superficial cancer lesions. This seems to be the appropriate field of treatment indication for imiquimod.

In conclusion, intralesional IL-2 achieved the best results for local tumor treatments of cutaneous metastases, particularly in stage III disease. More than 60% of complete remissions regarding the patients and more than 80% regarding the metastases were achieved. Its mode of action seems to be local and no distant responses have been described. This treatment is a good alternative, when surgical procedures become difficult. Topical DNCB and topical imiquimod are obviously inferior in their efficacy, and they cause additional discomfort for the patient. They both result in severe inflammatory reactions of the skin with itching and crusting. Therefore, they remain second choice in the local treatment of cutaneous metastases.

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50 Neoadjuvant Approaches in Melanoma

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Contents

ABSTRACT

Patients with clinically palpable regional lymph node metastases (AJCC stage IIIB-C) carry a risk of relapse and death that approaches 70% at 5 years. Surgical excision with complete regional lymph node dissection is the cornerstone of management, followed by adjuvant therapy with high-dose interferon-α2b (HDI). Neoadjuvant therapy has been demonstrated to improve outcome in the management of patients of multiple different solid tumors. In patients with melanoma, the quality of the host immune response differs between those with earlier and those with more advanced disease settings. Host immune tolerance is now understood to impede the results of therapy for advanced disease, but may be less an issue for patients with microscopic high-risk operable disease, where the host may be more susceptible to immunologic interventions. Phase II studies have shown that neoadjuvant biochemotherapy has limited activity in melanoma patients with local-regional metastases, where chemotherapy may potentially antagonize or alter the effects of immunotherapeutic agents. Studies of neoadjuvant HDI therapy for high-risk melanoma patients with bulky regional stage IIIB-C lymphadenopathy are ongoing and preliminary results have shown unexpectedly high clinical and pathologic response rates, without increased morbidity. Through the design of neoadjuvant trials in which it is possible to

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obtain biopsy samples before and after therapy, a greater understanding of the dynamic interaction between tumors and the immune system is possible. This should lead to the identification of new targets for the treatment of melanoma and aid the development of new immunotherapies that may have greater specificity and less toxicity. This will simplify the evaluation of promising new combinations of agents with HDI to build on the clinical, immunologic, and molecular effect of this therapy for patients with melanoma.

Key Words: melanoma; neoadjuvant; interferon; immunotherapy

1. INTRODUCTION

Survival of melanoma varies widely by stage from a potentially highly curable disease when detected in early stages, to a disease with dismal prognosis when it reaches advanced inoperable stages *(1)*. The American Joint Committee on Cancer (AJCC) divides cutaneous melanoma into four stages. Primary tumors confined to the skin without regional lymph node involvement are assigned stages I and II depending on the thickness (depth) of the tumor, ulceration of the overlying epithelium, or invasion of the reticular dermis or subcutaneous fat (Clark level IV or V). Stage III comprises a disease with clinical or pathological evidence of regional lymph node involvement, or the presence of in-transit or satellite metastases. Stage IV disease is defined by the presence of distant metastasis. Patients with stage I melanoma have an excellent prognosis with surgical treatment alone and a cure rate of more than 85%. The 3–5 years postsurgical relapse rate in patients with stages IIA and IIB is 20–30% and 40–55%, respectively. Stage III melanoma patients with regional lymph node involvement have a 5-year relapse rate of 60–80%, and stage IV disease continues to comprise a dismal prognosis with a median survival of only 6–9 months *(2,3)*.

Patients with clinically palpable regional lymph node metastases (AJCC stage IIIB-C) carry a risk of relapse and death that approaches 70% at 5 years *(1,4,5)*. Surgical excision with complete regional lymph node dissection is the cornerstone of management, followed by adjuvant therapy with high-dose interferon-α2b (HDI). HDI is the only form of adjuvant therapy that has ever shown a consistent, significant, and durable relapse-free survival benefit in multicenter randomized controlled trials of the US Cooperative Groups, that is universally agreed upon. Significant overall survival benefit of HDI has been shown in two multicenter randomized controlled trials *(6–8)*.

2. IMMUNITY AND IMMUNOTHERAPY IN MELANOMA AND IMPLICATIONS FOR ADJUVANT AND NEOADJUVANT THERAPY

Immunity to melanoma appears to be important for disease control in the adjuvant and advanced disease settings. Spontaneous regression has been reported in melanoma, suggesting a role for host immunity, indirectly supported by the presence of lymphoid infiltrates at primary melanoma associated with tumor regression. Host cellular immune response within melanoma has potential prognostic and predictive significance. T-cell infiltrates in primary melanoma are prognostic of disease outcome *(9)*, and T-cell infiltrates within regional nodal metastasis predict benefit from interferon (IFN)-α2b therapy *(10–12)*.

The quality of the host immune response differs between patients with earlier microscopic subclinical and those with more advanced clinically manifest disease. While T-helper type 1 (Th1)-type $CD4^+$ antitumor T-cell function appears critical to the induction and maintenance of antitumor cytotoxic T-lymphocyte (CTL) responses in vivo, and Th2- or Th3/Tr-type $CD4^+$ T-cell responses may subvert Th1-type cell-mediated immunity providing a microenvironment

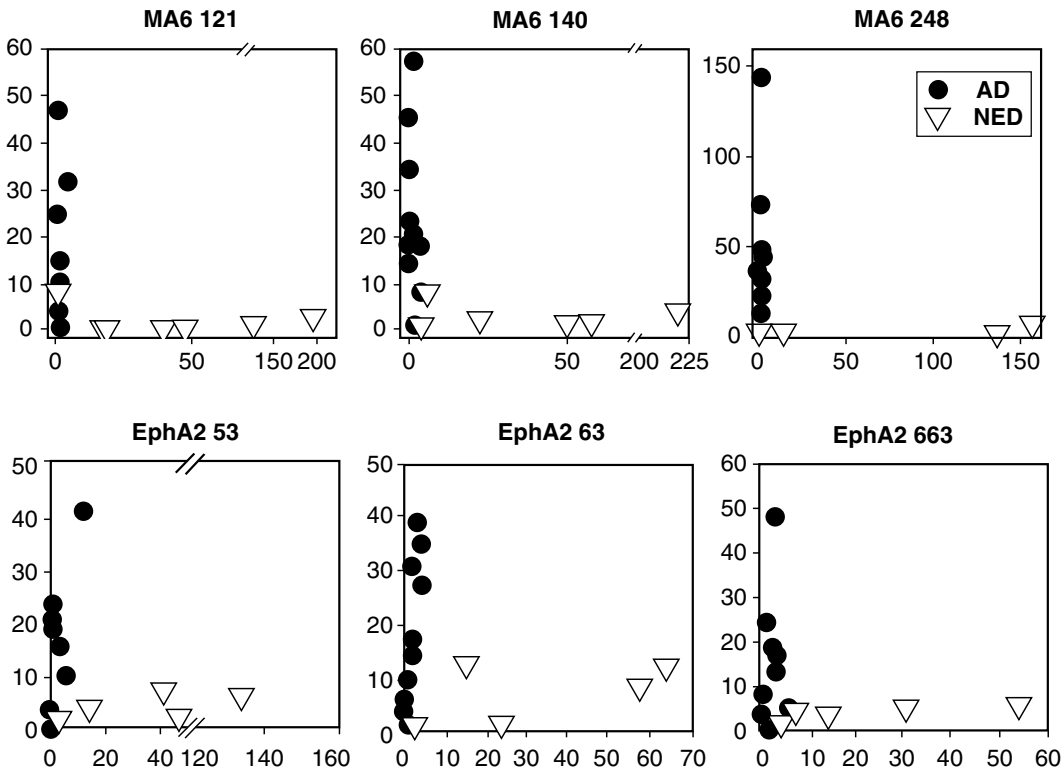

Fig. 1. Differential polarization of the immune response between the settings of active and inapparent disease. Patients with active stage IV melanoma display T_H2-type anti-MAGE-A6, anti-EphA2 responses, whereas patients with no evidence of disease exhibit T_H1-type immunity. *AD,* active disease; *NED,* no evidence of disease. *X*-axis, production of IFN-γ; *Y*-axis, production of interleukin-5.
Source: (Tatsumi T, Kierstead LS, Ranieri E, et al. Disease-associated bias in T helpher type 1 (Th1)/Th2 CD4(+) T cell responses against MAGE-6 in HLA-DRB10401(+) patients with renal cell carcinoma or melanoma. The Journal of experimental medicine 2002;196(5):619–28.)

conducive to disease progression, patients with active melanoma or renal cell carcinoma have been shown to display strong tumor antigen-specific Th2-type polarization. On the other hand, normal donors and patients who were disease free following therapy demonstrated either weak-mixed Th1-/Th2-type or strongly-polarized Th1-type responses to the same epitopes *(13)*. Therefore, factors of host immune tolerance that seem to impede advanced disease therapy may be less pronounced in the high-risk operable setting, where the host may be more susceptible to immunologic interventions Fig. 1.

3. ADJUVANT HIGH-DOSE IFN-α2B FOR HIGH-RISK RESECTED MELANOMA

Since 1984, three national cooperative group studies have evaluated the benefit of HDI as adjuvant therapy for resectable high-risk cutaneous melanoma. These included patients with regional lymph node metastases (T_{1-4}, N_1, M_0) and primary localized deep melanomas (T_4, N_0,M_0) that have a 5-year postsurgical relapse rate of more than 40–50%. The first and third of these studies both demonstrated significant overall survival prolongation, compared to observation (E1684) and compared to a vaccine (GMK) that was selected as the optimal vaccine candidate at the time (E1694). The second trial, E1690, was conducted in part before and in part after the

approval of HDI, and was associated with systematic crossover of patients from the observation-assigned arm to treatment with HDI at the event of nodal relapse. This trial showed differences in terms of relapse-free but not overall survival *(6–8)*. The treatment regimen comprised daily intravenous IFN-α2b at 20 MU/m^2 given for 5/7 days for 4 weeks followed by a self-administered subcutaneous phase of 10 MU/m^2 TIW for 48 weeks. Thus, the published literature contains three reports of relapse-free survival benefit and two reports of overall survival benefit with HDI therapy, a record that has yet to be equaled by any other therapy available for high-risk melanoma. These advantages of HDI amount to relapse frequency reductions of 24–38% and mortality reductions of 22–32% based upon the hazard ratios for patients treated with HDI or observation, or a previously promising ganglioside vaccine (GMK).

4. NEOADJUVANT TREATMENT OF POTENTIALLY RESECTABLE LOCAL-REGIONAL METASTASES OF CUTANEOUS MELANOMA

Neoadjuvant therapy has been demonstrated to improve outcome in the management of patients of multiple different solid tumors, including head and neck, breast, bladder, esophageal and rectal cancer. Benefits include improvements in survival, surgical resectability, local control, and organ preservation *(14–17)*. Another advantage of neoadjuvant therapy is the ability to evaluate the clinical and pathologic responses, and the potential to identify immunologic and histologic correlates of tumor response. Access to tumor tissue before and after neoadjuvant therapy may also allow a better understanding of the antitumor mechanisms of action that may enable more selective application of therapeutic agents to those patients who are more likely to benefit that would improve the therapeutic index and cost effectiveness of these agents.

5. NEOADJUVANT CONCURRENT BIOCHEMOTHERAPY IN MELANOMA PATIENTS WITH LOCAL-REGIONAL METASTASES

A phase II study of neoadjuvant concurrent biochemotherapy (BCT) in patients with potentially resectable local-regional metastases of cutaneous melanoma (stage III; nodal, satellite/in-transit metastases, and/or local recurrence) was conducted and reported by Gibbs et al. *(18)*. A total of 65 patients were treated with cisplatin 20 mg/m^2 intravenously (i.v.) on days 1–4, vinblastine 1.5 mg/m^2 i.v. on days 1–4, dacarbazine 800 mg/m^2 i.v. on day 1 only, interleukin-2 9 MIU/m^2 per day i.v. by 96 h continuous infusion on days 1–4, and interferon-α2a 5 MU/m^2 subcutaneously on days 1–5, repeated every 3 weeks. Patients underwent surgery after two to four courses of BCT. Patients with tumor regression after two preoperative courses received two additional postoperative courses. Of the 64 patients assessable for clinical response, 28 (44%) had a partial response. Of the 62 patients whose response was assessed histologically, 4 (6.5%) had no evidence of viable tumor in the surgical specimen (pathological complete remission, pCR) and 27 (43.5%) had a partial response, with an overall response rate of 50%. Tumor burden did not correlate with response, although patients who achieved a pCR had a significantly lower tumor burden ($p = 0.02$) *(19)*. In a second phase II study, two cycles of BCT were administered prior to and after complete lymph node dissection. Each cycle comprised cisplatin, 20 mg/m^2 i.v., on days 1–4; vinblastine, 1.6 mg/m^2 i.v., on days 1–4; dacarbazine, 800 mg/m^2 i.v., on day 1; interleukin-2, 9×10^6 IU/m^2/day i.v. over 24 hours, on days 1–4; and interferon-α, 5×10^6 IU/m^2/day subcutaneously, on days 1–5, every 3 weeks. Clinical responses were observed in 14 of 36 patients (38.9%) with measurable disease, including 13 partial responses (36.1%) and 1 complete response (2.8%). Complete pathologic responses were noted in 4 patients (11.1%). At a median follow-up of 31 months, 38 of the 48 patients (79.2%) were alive and 31 patients (64.6%) remained free of disease progression *(18)*.

These phase II studies indicated that neoadjuvant BCT is an active therapy for melanoma patients with local-regional metastases. However, BCT has failed to demonstrate superiority to chemotherapy alone in phase III randomized trials in patients with stage IV disease [Atkins (2003) and Keilhotz (2003)]. In addition, chemotherapy as single agents or in combinations has failed to demonstrate a survival advantage for patients with metastatic disease. IFN-α and IL-2 were administered at low (potentially suboptimal) dosage in these regimens, and in combination with chemotherapy there is the potential of interactions with chemotherapeutic agents, some of which have been shown to be immunosuppressive *(20)*, that may potentially antagonize or alter the therapeutic effects of IFN-α and IL-2.

6. NEOADJUVANT TREATMENT WITH HIGH-DOSE IFN-α2B FOR PATIENTS WITH REGIONAL NODAL PRESENTATION OR RECURRENCE AS STAGE IIIB-C MELANOMA

The efficacy of neoadjuvant HDI was investigated in melanoma patients with palpable regional lymph node metastases either presenting with clinical AJCC stage IIIB-C ($T_{any}N_{2,3}$) disease or with recurrent regional lymphadenopathy. In contrast to earlier BCT neoadjuvant studies employing lower dosages of IFN-α, HDI was administered in this study at the US Food and Drug Administration (US FDA)-approved adjuvant dosage regimen.

Patients with palpable regional lymph node metastases from melanoma (AJCC stages IIIB-C) underwent surgical biopsy at study entry and then received standard intravenous HDI (20 million units/m^2, 5 days per week) for 4 weeks followed by complete lymphadenectomy and standard maintenance subcutaneous HDI (10 million units/m^2 3 times per week) for 48 weeks (Fig. 2). Biopsy samples were obtained before and after intravenous HDI and subjected to immunohistochemical (IHC) analysis as well as routine pathologic study.

Twenty patients were enrolled, and biopsy samples were informative for 17. Eleven patients (55%) demonstrated objective clinical response, and 3 patients (15%) had complete pathologic response. At a median follow-up of 18.5 months (range, 7–50 months) 10 patients had no evidence of recurrent disease. By comparison, in the setting of advanced stage IV disease, response rates of less than 20% were achieved, although a number of patients had durable responses ranging from 26 to more than 30 months *(21)*.

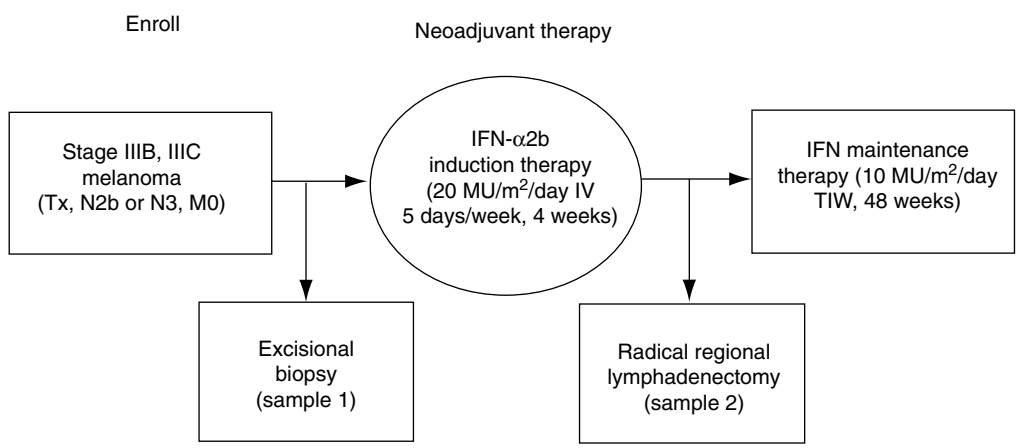

Fig. 2. Neoadjuvant high-dose interferon-α2b (HDI) treatment schema. Tumor biopsies were obtained before and immediately after the induction phase of HDI. IV, intravenous; IFN, interferon-α; TIW, 3 times per week.

IHC analysis of tumor tissue revealed that HDI did not appear to influence the tumor cell phenotype, proliferation rate, or apoptotic fraction of cells in tumor biopsies, and did not significantly affect tumor vasculature, irrespective of clinical response. In contrast, clinical responders had significantly greater increases in endotumoral $CD11c^+$ and $CD3^+$ cells and significantly greater decreases in endotumoral $CD83^+$ cells compared with nonresponders. Correlations were also shown between clinical response and modulation by neoadjuvant IFN-α2b of the signaling pathway molecules phospho-STAT1 (upregulated) and phospho-STAT3 (downregulated). IFN-α is capable of inducing changes in STAT1 activation in the host, and this is the major pathway through which IFN-α mediates its indirect antitumor effect *(22,23)*. STAT3 activation is a constitutive phenomenon in a number of solid tumors, including melanoma where it is now thought that this is part of the process that leads to immunological tolerance *(24,25)*. Neoadjuvant IFN-α2b was shown to significantly induce the expression of transporters associated with antigen processing 2 (TAP2) in tumor and lymphoid cells *(26)*. Mutations in TAP-1 and TAP-2 required for the transport of cytosolic endogenous peptides to the endoplasmic reticulum correlate with increased metastatic potential and reduced host survival in several malignancies.

7. CONCLUSION

Neoadjuvant therapy allows new insights into melanoma and its biological and immunologic response to therapeutic interventions, such as high-dose IFN-α2b. Neoadjuvant HDI therapy for high-risk melanoma patients with bulky regional stage IIIB-C lymphadenopathy results in high clinical and pathologic response rates without increased morbidity. Through the design of neoadjuvant trials in which it is possible to obtain biopsy samples, a greater understanding of the dynamic interaction between tumors and the immune system is possible. This should lead to the identification of new targets for the treatment of melanoma and aid the development of new immunotherapies that may have greater specificity and less toxicity. It is now possible to examine the effects of candidate new therapies in advanced metastatic disease, and the neoadjuvant setting, for patients who have regional nodal metastatic disease. The latter setting has shown improved benefit from high-dose IFN-α2b. If therapy with only 1 month (4 weeks) of i.v. high-dose IFN-α2b is effective, this will simplify the evaluation of promising new combinations of agents with high-dose IFN-α2b to build on the clinical, immunologic, and molecular effect of this therapy for patients with melanoma.

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51 Implications of Chemo/Biochemotherapy in the Treatment of Metastatic Melanoma

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CONTENTS

ABSTRACT

Metastatic melanoma is a devastating disease, and therapeutic options are few and have not shown a survival impact in randomized trials. Single agent and combination cytotoxic chemotherapy approaches have had limited therapeutic effect. Immunotherapy with high-dose interleukin-2 (IL-2) has produced long-term remissions in selected patients, but its toxicity and expense have hindered its widespread application. Lowering the dose of IL-2 and combining it with other agents is a strategy termed biochemotherapy. Although apparently successful in phase II trials, randomized trials testing this approach against chemotherapy alone have not produced statistically significant improvements in response or survival. Clearly, metastatic melanoma represents an area of major unmet need.

Key Words: Immunotherapy; chemotherapy; biochemotherapy; metastatic melanoma

1. INTRODUCTION

Metastatic melanoma is a devastating disease with a rising incidence in the USA with more than 8000 deaths in 2007 *(1)*. The dismal prognosis for those afflicted is underscored by the fact that the number of newly diagnosed stage IV patients per year closely mirrors the annual death rate. Treatment options for patients with metastatic melanoma are few and largely ineffective making clinical trials the most attractive therapeutic option. This chapter discusses the role of chemotherapy, immunotherapy, and combined biochemotherapy (chemoimmunotherapy) in this disease.

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2. CYTOTOXIC CHEMOTHERAPY

The use of cytotoxic chemotherapy as a therapeutic modality for metastatic melanoma goes back several decades. However, even in 2008, dacarbazine (DTIC) remains the only FDA-approved cytotoxic agent and no other drug or combination has proved superior in prospective, randomized trials. About 7.5% of patients with metastatic melanoma have a RECIST-defined response to this agent *(2)*, a number considerably lower than historical data would suggest *(3)*. Another agent widely used in place of DTIC is temozolomide, an imidazotetrazine derivative that is in fact converted to the same active metabolite. The only reported randomized trial comparing these two agents showed similar response rates and survival *(4)*, but the convenience of oral administration and its modest efficacy in central nervous system metastases *(5)* have made this a popular choice despite lack of endorsement from the FDA.

The premise behind combination therapy is the potential for additive or synergistic effects using agents with nonoverlapping toxicities. This approach has been ineffective in metastatic melanoma and DTIC has yet to be "beaten" in a randomized trial *(6–8)*. Indeed, based on results of these randomized studies the routine use of combination chemotherapy for melanoma can no longer be justified.

3. IMMUNOTHERAPY

Immunotherapy has been an approach tested and used in metastatic melanoma for many years based on observations of tumor infiltration by cytotoxic T lymphocytes and spontaneous regressions of primary and metastatic sites of disease. Responses to interleukin-2 (IL-2), a cytokine released by activated T cells, are well documented and the use of the NCI-developed high-dose bolus regimen is associated with long-term remissions in a few, selected patients *(9)*. This regimen is FDA-approved for patients with metastatic melanoma, but there are several hurdles to its use including the need for specialized training, intensive support, and high cost which has restricted its use to a handful of centers around the country. Lower doses of IL-2 are administered subcutaneously, while more practical, less toxic, and less expensive have low response rates and durability and are no longer recommended *(10)*.

4. BIOCHEMOTHERAPY

A "happy medium" would be the use of intermediate doses of IL-2 administered by intravenous infusion, and this has been combined with multiple chemotherapy agents in various biochemotherapy regimens. The most widely used and tested of these is the combination of CVD (cisplatin, vinblastine, and DTIC) plus IL-2 (continuous infusion at 9 MIU/m^2) and low-dose, subcutaneous interferon-α (IFN-α) either sequentially (CVD followed by IL-2 and IFN-α) or concurrently *(11,12)*. In phase II trials, the concurrent regimen was shown to be more practical and less toxic *(13)*. However, randomized phase III trials testing this combination as compared with chemotherapy alone have not shown superior results in response rates or survival *(14–16)*. Recent metaanalyses of biochemotherapy combinations indicate that while response rates may be slightly higher for biochemotherapy, survival is not improved *(17,18)*.

Overall, results with IL-2 suggest that in metastatic melanoma, the only regimen that may have some efficacy and produces durable benefit is the high-dose bolus regimen. A recent publication of the use of high-dose bolus IL-2 in the setting of previous failure of CVD-based biochemotherapy is enlightening in this regard *(19)*. The 20% response rate with two long-term durable remissions seen in this trial implies a dose–response relationship with IL-2 in melanoma similar to that seen in renal cell carcinoma.

5. SUMMARY AND CONCLUSIONS

Traditional approaches to tackling the problem of metastatic melanoma have focused on chemotherapy (single agents and combinations), immunotherapy, and combined biochemotherapy. After decades of research it is clear that in terms of cytotoxic chemotherapy, single agents are as effective as combinations and DTIC remains (a poor) therapeutic standard. In the immunotherapy arena, the best choice is the use of high-dose bolus IL-2, but this is a treatment that is applicable only to selected patients at selected institutions. Lowering the dose of IL-2 in an attempt to make it more palatable and combining it with other agents has not proved to be a useful therapeutic strategy in randomized trials. Indeed at this time, it is even more than ever appropriate to offer all patients with metastatic melanoma the opportunity to participate in a clinical trial.

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XIV Future Perspectives

52 Future Perspectives for Cancer Metastasis: Unanswered Questions and Unquestioned Answers

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CONTENTS

ABSTRACT

In this sentinel lymph node (SLN) era, two new paradigms of cancer metastasis have emerged. First, in general, cancer spreads in a progressive fashion from the primary site to the SLNs and beyond to distant sites. Second, cancer heterogeneity may have a genetic basis. Gene profiling of cancer could identify subgroups of cancer patients for tailored, personalized therapy. With this knowledge and new insights, ever more unanswered questions and unquestioned answers on cancer metastasis have arisen. These will be the focus of the upcoming 3rd International Symposium on Cancer Metastasis and the Lymphovascular System: Basis for Rational Therapy, May 7–9, 2009, in San Francisco.

Key Words: cancer metastasis; lymphovascular system; ignorance

1. INTRODUCTION

According to the American Cancer Society, approximately 1.5 million people in the USA are diagnosed with solid organ cancer yearly and about 50% of these yearly will die of cancer due to metastases *(1)*. Although a tremendous effort has been expended in the treatment of metastatic cancer, the overall result is only modest in some cancers but marginal in most. Over the past decade, two major paradigms have evolved that take a fresh look at the process of cancer metastasis. First, the development of the sentinel lymph node (SLN) concept that cancer, in general, spreads in an orderly fashion from local invasion to the regional SLN prior to widespread dissemination. For example, using the melanoma and breast cancer models from the SLN

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era, it has been shown that about 80% of the time, metastasis from the primary site follows an orderly pattern of progression through the lymphatic network initially, whereas about 20% of the time, systemic metastasis occurs without evidence of lymphatic invasion (although these, too, may originate from the lymphatic route of transport). Thus, the spectrum theory *(2,3)* that the spread of cancer cells is progressive with regional lymph node involvement and later seeding of systemic sites, has been validated. Second, the mechanism of cancer heterogeneity may be due to differences in gene structures and expression. Thus, gene profiling may identify a subgroup of patients for individualized, tailored therapy.

2. CANCER METASTASIS AND THE LYMPHOVASCULAR SYSTEM

As the aforementioned paradigms were being formulated to describe the process of cancer growth and metastasis, it was timely for the 1st International Symposia on Cancer Metastasis and the Lymphovascular System, held in May 2005 in San Francisco *(4,5)*, to trace the sequence of events from initial malignant growth to clinically evident cancer. The clinical patterns of cancer metastasis are well documented for many different types of cancer. Yet the precise molecular mechanisms linking each step from initiation of the cancer within the tissue microenvironment to invasion into the lymphatic or blood vascular systems and widespread dissemination remain elusive. Therefore, the 2nd International Symposia on Cancer Metastasis and the Lymphovascular System, held in May 2007 in San Francisco, further addressed these proximate cellular and molecular mechanisms within the translational context of clinical perspectives of cancer metastasis as delineated in this volume's Preface. The 2nd International Symposium forms the basis of this book, From Local Invasion to Metastatic Cancer: Involvement of Distant Sites through the Lymphovascular System. Although each session in the symposium is independent of each other, these sessions are linked together to illustrate how important it is for us to understand cancer metastasis from its primary growth within the tumor microenvironment and its progression to metastatic potential to the distant sites through the lymphovascular system, oftentimes, in an orderly fashion via the gateway of the SLN(s). The basis of this progression is genetically determined. Thus, it is important to scrutinize the molecular mechanisms of metastasis to understand the complexity of cancer metastasis. The underlined theme is that cancer metastasis is progressive. The most optimal time to eradicate the cancer is at its early stage of development. As cancer is heterogeneous with respect to its biological characteristics, different subgroups cancer patients may be selectively identified by genetic profiling with specific biomarkers so that more rational therapy can be tailored to different subgroups of patients (Fig. 1). From the symposium panel discussions and book chapters, we have formulated the following list, albeit far from complete, of unanswered questions so that new hypotheses may be developed to be scrutinized by further research investigators and clinical trials to reach new conclusions (Fig. 1).

3. UNANSWERED QUESTIONS AND UNQUESTIONED ANSWERS

We plan to tackle these important questions in future conferences in this series:

1) During active proliferation of cancer in the primary site, how do cancer cells acquire the mobility and molecular characteristics to invade the lymphatic channels and/or blood vasculature? Through a better understanding of lymphangiogenesis and hemangiogenesis, what are the molecular signals and pathways that determine the selectivity of lymphatic versus blood vascular invasion? *(6)*

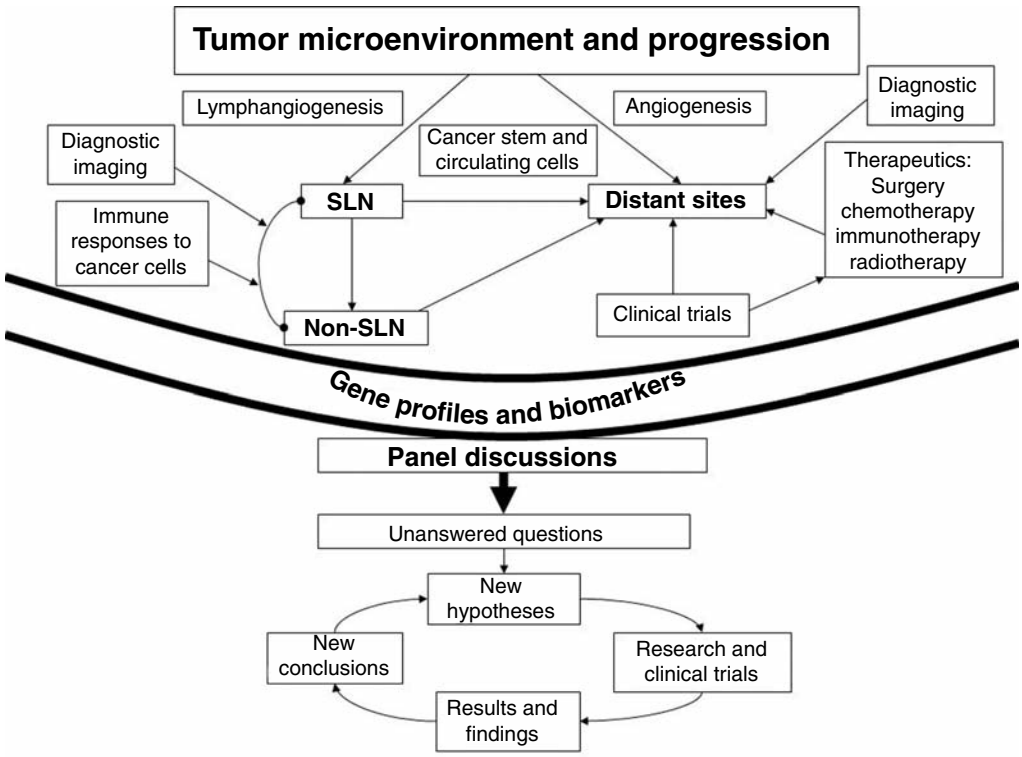

Fig. 1. Summary overview of all sessions held in the 2nd International Symposium on Cancer Metastasis and the Lymphovascular System: Basis for Rational Therapy from May 2 to 5, 2007, in San Francisco. These sessions demonstrated that cancer metastasis is a progressive process from the primary site to the distant sites, oftentimes via the gateway of the sentinel lymph nodes. Rational therapy is based on a clear understanding of the mechanisms of cancer metastasis through the lymphovascular system. Genetic profiling and specific biomarkers can be used to separate different subgroups of cancer patients. Unanswered questions from the panel discussions have generated new hypotheses for future basic science investigations and clinical trials.

2) During invasion of the lymphovascular system, do the cancer cells first enter the lymphatic stream and arrive at the SLNs, which then serve as the gateway to systemic metastasis? Under which circumstance do the cancer cells continue through the lymphovascular system simultaneously and subsequently reach the bloodstream for dissemination?
3) When cancer cells spread through the lymphatic system, are passive processes involved? What are the influences of mechanical factors and anatomical structures on the facilitation of cancer cell transport through the lymphatic channels? If an active process is operative, what are the signaling mechanisms between the endothelial cell receptor and cancer cell surface molecules that facilitate or block cancer cell entry into the lymphatic channels and physically how and where exactly do they enter?
4) What is the role of cancer stem cells? If cancer stem cells do exist *(7–9)* how do they spread through the lymphovascular system? What are the phenotypic, genetic, and molecular profiles of cancer stem cells so that they can be selectively targeted? What is their relation to stem cells that might be involved in generating the tumor vasculature?
5) How can the route of cancer metastasis be tracked accurately by new developments in cancer imaging? Can molecular imaging of cancer specific growth pathways be developed to identify cancer cells more selectively and to selectively intervene therapeutically in cancer growth? If

early metastatic deposits are discovered, can new radiotherapeutic techniques such as gamma knife approaches be used to eliminate or suppress these deposits without surgical interventions?

6) What are the host factors that keep the cancer dormant? How do dormant cancer cells surge to become aggressive and uncontrolled?
7) With the development of new molecular techniques, should an effort be made to develop molecular taxonomy to stage and subgroup cancer patients more accurately than the current clinical and histological databases? Is there a place for "surgicogenomics" and "radiogenomics" alongside pharmacogenomics in future cancer treatment?
8) Will favorable clinical trial results for the treatment of metastatic cancer provide the insights needed to identify therapeutic agents in adjuvant therapy for high-risk patients following definitive surgical resection?

These challenging issues will be highlighted and debated by world experts and promising new investigators in the 3rd International Symposium on Cancer Metastasis and the Lymphovascular System: Basis for Rational Therapy to be held in San Francisco, May 6–9, 2009. The mission of this upcoming symposium is to bring together again multidisciplinary basic and clinical scientists from the USA and abroad to address and translate these important unresolved issues of cancer metastasis.

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