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About the art in this issue:
Craig Damlo is an innovation expert specializing in creating a culture of innovation and design thinking. With a background in aerospace product and innovation development, Craig helps companies understand how to best utilize their resources in order to maximize innovation within their own groups. Craig has always been interested in how things work and problem solving, constantly taking things apart as a child, and this desire drove him to use photography as a way to capture what he was observing around him. He uses his knowledge of physics to tell a photographic story of how time and movement affect image capture and how the passing of time—as well as the human hand—works to reclaim objects, as showcased in this collection. Craig also uses photography to hone his innovation skills through careful, patient observation and the capture of still moments to observe just how things operate. Learn more about Craig and his work at Getty Images, www.flickr.com/craigdamlo, and www.soapboxrocket.com.

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Personalizing the Attack on Cancer: Zeroing in on Targets

The “War on Cancer” continues with some recent encouraging victories.

Advances in genomics and other laboratory-based diagnostic technologies have transformed our understanding of the fundamental molecular basis of cancer, and applying this knowledge has led to new generations of targeted therapeutics. Pathologists are at the center of this revolution of personalized medicine as they are faced with the challenges of deploying emerging technologies into routine patient care. In addition, pathologists are grappling with the challenge of combining molecular knowledge with long-established systems of tumor classification based on light microscopy.

Unfortunately, pathology practices are often also battling diminishing reimbursements and increasing regulatory burdens. The latest technologies are often considered “research use only,” forcing clinical laboratories into complex in-house validation processes to qualify their use under the Clinical Laboratory Improvement Amendments as laboratory-developed tests (LDTs). Frequently, third-party payers deny coverage of new diagnostic technologies, putting some laboratories out of business. Further complicating matters is the intention of the US Food and Drug Administration to more closely scrutinize hospital laboratory-developed LDTs.

Despite these challenges, there is no question that molecular analysis will become routine and fundamental to the diagnosis and management of cancer.

The application of the knowledge derived from the completion of the Human Genome Project and the rapid collapse in costs for deep genetic and transcriptomic analysis are fundamentally transforming the way pathologists understand and classify cancer and will certainly impact the way oncologists manage and treat malignancies in their patients.

In this issue of Cancer Control, members of the anatomical and clinical pathology departments of the H. Lee Moffitt Cancer Center & Research Institute review several topics of interest to oncologists and other health care professionals, focusing on the transformative effects of molecular pathology on the contemporary practice of pathology.

Dr Saeed-Vafa and I present an update on developments in digital and analytical microscopy. Light microscopy has been the fundamental tool for pathologists to analyze cancer for more than 100 years and has provided the knowledge underlying the classical classification of malignancy based on the organ and cells of origin along with the assessment of biological appearance, which is used for grading. Microscopic examination of surrounding tissues and lymph nodes create the current staging systems.

Advances in analytics, staining reagents, digital microscopy, and computer-assisted vision have created new opportunities to analyze tissue to extract more detailed information, including more precise measurements of prognostic and predictive biomarkers, such as protein and nucleic acids. Improvements in digital image analysis will help reduce errors in pathology and provide more accurate information for treatment selection. To this point, Dr Zota and I review key advances in the analytical methods of molecular biology that recently transformed pathology. The ability to accurately analyze key mutations in driver mutations such as EGFR in lung cancer and BRAF in melanoma from routinely acquired tissue specimens has enabled personalized medicine and targeted therapies to be routinely implemented.

The decreased costs of next-generation sequencing now enable hundreds of genes — up to whole genomes — to be cost-effectively analyzed in the hospital laboratory. In fact, one of the greatest current challenges is the management of “information overload.” The so-called “long tail” of cancer mutations illustrates that cancer is a diverse disease characterized by a multitude of diverse driver mutations and a degree of genomic liquidity that results in varied genomic “landscapes” and evolutionary “trees” within tumors. Some driver mutations occur in small numbers of tumors, making it difficult to conduct conventional, large-scale clinical trials. This fact has led to the creation of basket-type trials in which tumors are analyzed and then matched to selective therapeutic inhibitors.

We now conceptualize cancer as a 4-dimensional disease in a patient that evolves over time with populations of malignant cells that react, migrate, and adapt to the therapies oncologists unleash on them. We have also learned that some mutational drivers and events cross anatomical locations, forcing health care professionals to consider novel molecular classifications that transcend conventional histological ones.

On a positive note, the deployment of novel tools, such as digital polymerase chain reaction, will help
us measure trace amounts of biomarkers in biosamples, such as blood and urine, potentially allowing more accurate screening, monitoring, and effective treatment selection, including the discontinuation of medically futile and toxic therapies and the switch to more effective ones.

Furthermore, the advances in immunotherapy are challenging pathologists to provide new, accurate, and predictive biomarkers to improve patient selection and understand response. Some of these biomarkers may include analysis of the complex composition of the tumor microenvironment.

The paper by Dr Valderrabano and colleagues illustrates the impact of molecular analysis on conventional optical microscopy for the assessment of thyroid lesions. The accurate presurgical analysis and classification of thyroid lesions are essential to avoid unnecessary morbidity. Conventional cytopathology has difficulty distinguishing certain benign from malignant lesions based on light microscopy, thus leading to frequent overtreatment in some patients and possible undertreatment in others. The identification and routine deployment of molecular markers to improve classification of indeterminate lesions will be of great benefit to patients and surgeons and will reduce costs to the health care system by limiting unnecessary surgical procedures.

Researchers in the field of molecular technologies have made inroads into the diagnosis and management of malignant hematological conditions. Dr Hussaini reviews current trends in hematological malignancy testing. These methods and approaches often foreshadow similar advances in the approach to solid tumors and provide a roadmap for effectively implementing novel technology platforms.

Dr Cáceres and others introduce us to the concept of the “liquid biopsy” and circulating tumor cells (CTCs). CTCs hold promise for the sequential temporal examination of malignancy prior to, during, and after therapy. CTC technologies coupled with other molecular methods to analyze cell-free DNA in blood are expected to provide us with tools to better select therapy for metastatic disease and enable health care professionals to determine earlier if a particular therapy is succeeding or failing, thus allowing for the potential real-time modulation of treatment strategies. Further refinements to the sensitivity rates of liquid-biopsy technologies are also expected to lead to improved screening and the earlier detection of cancer, improved molecular-based staging, and novel and practical methods to monitor minimal residual disease.

Drs Zibadi and Coppola review the complex challenges of diagnosing and managing Barrett esophagus, which represents a problematic lesion that can progress to malignancy. Use of novel molecular testing methods may enable us to better manage this difficult clinical condition and avoid overtreatment and overscreening.

Drs Henderson-Jackson and Bui review new developments in the area of soft-tissue neoplasms and the application of molecular classification approaches to improve the classification of these heterogeneous lesions and, perhaps more importantly, to uncover therapeutically useful targets that could be used for treatment.

Drs Khalil and Altiok review how the treatment course for patients with lung cancer has been altered in recent years with the uncovering of the role of key molecular drivers, including EGFR mutation, in a subset of cases. The uncovering of these common mutations in lung cancer has expanded the demand for the routine molecular testing of solid tumors and provided the impetus for routine solid tumor molecular diagnostic laboratories in many specialized hospitals. In addition, the study of acquired resistance mechanisms has uncovered new insights into how tumors evolve and evade personalized treatment options, opening up new strategies for overcoming these resistance mechanisms.

Dr Macaulay discusses how advances in complex molecular testing have significantly impacted the subspecialty of neuropathology and the classification and understanding of brain tumors. The uncovering of driver mutations in IDH1 and other molecular events, including chromosomal alterations and DNA epigenetic events, have driven the development of routine molecular analysis of brain cancer to improve the classification of tumors and drive treatment selection.

In an article by Dr Rosa and coauthors regarding the overexpression of vascular endothelial growth factor A in invasive micropapillary colorectal carcinoma, we are reminded that immunohistochemistry is a molecular technology — essentially “in situ proteomics” — that enables us to evaluate the molecular characteristics of histological structures. Dr Rosa and colleagues show that spaces originally thought to be “preparation artifacts” are actually neovascular structures. This aspect of the biology of micropapillary carcinoma may explain its clinical aggressiveness and potentially offer new therapeutic targets.

Breast cancer is one of the strongest examples of how molecular methods have transformed our understanding of the pathology and classification of this disease. Historically, classification was exclusively based on microscopic examination. However, this classification has now been transformed with extensive basic, translational, and clinical research, resulting in a new molecular understanding of breast cancer. The molecular analysis of breast cancer has uncovered “molecular portraits” that help define distinct subtypes of the disease with different clinical behav-
iors that each require different treatment strategies. In 2 papers, Drs Rosa and Khazai review current methods and trends in the molecular classification of breast cancer and the implications for the improved, enhanced quality of treatment and better outcome for patients.

Classical pathology methods typically require “fixing” tumor tissue, which essentially is killing and preserving the cancer as a frozen moment in time. Although this method has yielded spectacular dividends, it is still a “snapshot” of a very dynamic living system composed of a complex mixture of tissue types, including malignant cells, and a variety of host cells. Drs Kreahling and Altiok discuss alternate methods to maintain cancer cells ex vivo that may enable the detailed evaluation of drug-sensitivity profiles, thus arming health care professionals with the ability to select more effective treatments for their patients.

Also included in the April issue of Cancer Control is original research from Dr Mahipal and colleagues presenting data on the effect of age on clinical outcomes among patients enrolled in phase 1 clinical trials at Moffitt Cancer Center. Dr Grigg-Gutierrez and others share a case report on primary enteropathy-associated T-cell lymphoma type 2. In 2 Special Reports, Mr Patel and Dr Kilgore provide a systematic review of the cost effectiveness of colorectal cancer screening strategies, and Dr Lu and colleagues discuss the risk of colorectal cancer by subsite in a nationwide prostate cancer cohort in Sweden.

Pathology has been profoundly affected by these changes and pathologists — frequently unrecognized by patients — serve as critical members of the health care team. Pathologists remain key to deploying new diagnostic molecular technologies in the ongoing battle against cancer, providing the coordinates and advanced surveillance required for correctly aiming the powerful next generation of targeted therapeutic weaponry in the ongoing war.

In summary, we are living in revolutionary times in terms of how we understand, diagnose, and treat cancer. I hope you enjoy and benefit from reading this issue of Cancer Control.

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The Department of Anatomic Pathology at the H. Lee Moffitt Cancer Center & Research Institute is an academic department that participates in the teaching of medical students, residents, and anatomical pathology fellows in its accredited fellowship programs. Research is also a significant component of the academic mission of the department and its pathologists are engaged in collaborating with other Moffitt Cancer Center investigators and researchers on clinical trials and the publication of study results. One of the main focuses of the department has been the identification and application of new prognostic markers for the analysis of solid tumors. This includes an extensive immunohistochemistry laboratory, an advanced molecular diagnostics facility that recently introduced clinical next-generation sequencing, digital microscopy, and technologies for the capture and analysis of circulating tumor cells. The pathologists also work with other Moffitt programs in creating algorithmic pathways that provide a standardized tool to guide patient care and treatment plans as well as translate new discoveries into clinical diagnostics. New testing and methodologies are continuously being added.

For more information about the Department of Anatomic Pathology, please call 813-745-3741 (during normal business hours).

www.MOFFITT.org
Digital pathology will continue to advance and provide precise prognostic information and help guide treatment decisions.

Practical Applications of Digital Pathology
Daryoush Saeed-Vafa, MD, and Anthony M. Magliocco, MD

Background: Virtual microscopy and advances in machine learning have paved the way for the ever-expanding field of digital pathology. Multiple image-based computing environments capable of performing automated quantitative and morphological analyses are the foundation on which digital pathology is built.

Methods: The applications for digital pathology in the clinical setting are numerous and are explored along with the digital software environments themselves, as well as the different analytical modalities specific to digital pathology. Prospective studies, case-control analyses, meta-analyses, and detailed descriptions of software environments were explored that pertained to digital pathology and its use in the clinical setting.

Results: Many different software environments have advanced platforms capable of improving digital pathology and potentially influencing clinical decisions.

Conclusions: The potential of digital pathology is vast, particularly with the introduction of numerous software environments available for use. With all the digital pathology tools available as well as those in development, the field will continue to advance, particularly in the era of personalized medicine, providing health care professionals with more precise prognostic information as well as helping them guide treatment decisions.

Introduction
Advances in molecular biology are transforming image-based specialties like pathology from primarily subjective-based analyses to quantitative-based digital analyses. The technologies used to capture high-resolution images, create digital versions of glass slides, and make virtual microscopy possible, as well as advance machine-based learning, have paved the way for the digital era of pathology. This has also led to the development of multiple, image-based computing environments capable of quantitative and morphological analyses. It is possible that we may see automated systems in the future assisting pathologists in examining tissue by performing some level of triage, similar to what has been done with the Papanicolaou test in cytopathology. At the time of publication, several digital software products have been developed, including AQUA (HistoRx, New Haven, Connecticut), Tissue Studio (Definiens, Munich, Germany), HALO (PathXL, Belfast, Northern Ireland), ImageJ (National Institutes of Health, Bethesda, Maryland), and many others with analytical capabilities that could be applied to digital pathology images.

Digital Image Analysis
The key area in which digital microscopy has an ad-
vant advantage over a human observer — even an expert — is in the precise quantification of the features in a digital image. This might include counting features in a defined area, such as nuclei or mitotic figures, measuring precise distances, or recording the intensity of a stain in defined cell populations. The analysis is only as good as the quality of the algorithms for isolating the feature of interest (eg, separating malignant nuclei from benign ones) or mapping areas of cancer from surrounding normal areas. In addition, machine algorithms may be stymied by preanalytical variables (eg, inadequate fixation, necrosis, warm ischemia). However, the ability of computers to measure features with great precision lends itself to applications such as screening large numbers of slides for small areas of abnormality, precisely quantifying important features in malignant cells (eg, proliferative rate or presence of tumor-associated lymphocytes), and quantifying important protein markers used in therapy selection (eg, tissue levels of estrogen receptor in breast cancer). With emerging interest in immunotherapies, computer-assisted approaches are being developed to better quantify the immune cell components of the microenvironment. More sophisticated analyses include the development of artificial intelligence or machine learning capable of recognizing key morphological features in tissue sections.

**AQUA**

In 2005, McCabe et al\(^1\) developed an approach for AQUA using immunofluorescent-stained slides. Determining protein expression via traditional immunohistochemistry (IHC) is a technique that allows the direct visualization of protein expression in tissue sections in situ. However, IHC has many disadvantages, including the subjectivity of interpretation due to a variety of reasons (eg, interobserver variability and variability from one assay to another).\(^2\) Despite its limitations, IHC is useful and is standard in the surgical-pathology laboratory and most hospitals. Digital analysis of fluorescent-labeled, IHC-stained tissue specimens may make it possible to address some of the limitations inherent in classical chromogenic IHC. The dynamic range for immunofluorescent labeling is far larger than for standard insoluble product assays, thus allowing for more precise quantification. In addition, the ability to stain the same tissue for multiple targets, multiplexing by using different fluorescent labels, opens up the possibility of isolating subcomponents of tissue (eg, nuclei, keratin-positive cells) and potentially normalizing quantification across different assay runs.

AQUA is based on these key features of fluorescent IHC.\(^2,3\) It analyzes digital images captured from slides stained with the typical immunofluorescence protocol in which an unlabeled primary antibody targets a molecule and then a secondary antibody coupled to a fluorophore (a fluorescent chemical compound that can re-emit light if it is excited) recognizes and binds to the primary antibody. Using different antibodies and fluorophores, different compartments and targets can be specifically marked. Typically, a digital camera will capture sequential images of the multiplex-stained tissue by capturing an exposure through successive filters, thus isolating each of the target probes. AQUA then creates tissue “masks” defined by specifically stained features. For example, the diamidino-phenylindole stain will identify nuclei, so AQUA can be used to define a nuclear mask, which can then be used to isolate the key pixels where the target antibody signal is captured. Combinations of masks can be created and arranged so that signal intensity in various tissue components (eg, estrogen receptor within nuclei of keratin-positive cells) can be digitally isolated and precisely quantified. The technology then provides a score for the particular target in any area of interest (Figs 1 and 2A–C). This AQUA score is calculated from the sum of the target pixel intensity divided by the compartment area. All AQUA scores are also normalized for image exposure times and are directly proportional to molecules per unit area or protein concentration.\(^2\) Providing an objective and quantitative method for determining protein expression has applications in the research setting and clinical practice in terms of providing precise information regarding the subcellular location and quantity or concentration of a particular antigen — quantities that may have important implications regarding the
effectiveness of a particular therapy. AQUA scores are reproducible and provide continuous variable data that can be used to define superior marker classifiers and improve the prognostic and predictive perfor-

mance of in situ–based tissue biomarkers.

**Tissue Studio**

Tissue Studio is an image analysis environment that takes a context-based, relational approach, rather than a pixel-based, pattern recognition approach. Its segmentation algorithms begin by grouping pixels into objects called image object primitives. These objects are then merged into what the user recognizes as the microanatomy of the tissue and are then related to the super objects (eg, white space, lumen, tissue, background, glands). Because of this hierarchical relationship, which links the smallest elements in an image to the largest, Tissue Studio provides a means for detecting and quantifying hundreds of relational attributes of the tissue that could be related to clinical outcome. Doing so enables pathologists to ask more advanced questions of the underlying biology.

A novel example demonstrating the power of this tool came from Beck et al\(^1\) in which the researchers used the Developer XD (Definiens) image analysis environment to create a processing pipeline consisting of basic image processing and feature construction, training and application of the epithelium/stroma classifier, and construction of higher-level features to create a computational pathologist (C-Path) to analyze microscopic images taken from patients with breast cancer. C-Path automatically quantified morphological features derived from the images to create a prognostic model.\(^4\) The researchers discovered that the score of the prognostic model was strongly associated with overall survival rates and was independent of clinical, pathological, and molecular factors.\(^4\) Their analysis was not predicated on features predefined by an expert pathologist; thus, their image analysis system was mainly automated and had no manual steps.\(^4\) Furthermore, C-Path identified features in the stroma as being better predictors of patient survival rates than the conventional use of the tumor epithelial cells themselves.\(^4\) That stromal features provided statistically significant prognostic information was a novel finding because morphological characteristics of the stroma had not been previously identified by pathologists as providing clinically significant information.\(^4\)

Another example of the utility of this software came in 2013 when Definiens and Metamark Genetics (Cambridge, Massachusetts) agreed to use the image analysis platform for a dual-marketed, multiplex, immunofluorescence prognostic test for prostate cancer.\(^5\) The companies claim that the technology they marketed together automatically classifies tumors of the prostate as “regions of interest” and will quantify the expression of proteomic markers in biopsy specimens.\(^5\) This technology is
likely to help health care professionals make better informed treatment decisions.

**Fractal Analysis**

Mandelbrot introduced the concept of fractals in 1967. A fractal is a complex, never-ending pattern self-similar across different scales and generated by a process in a recursive feedback loop. Formally, fractals are defined by measurements of their shape, such as the degree of their boundary fragmentation or their irregularity across multiple scales. Fractal analysis can characterize irregular structures that maintain a constant level of complexity across a range of scales. A practical application of fractal analysis in the field of medicine is analysis of the morphological complexity of tumors. For example, an automated fractal analysis technique has been used to quantify the morphological complexity of breast epithelium and prostate cancer, and the morphological complexity of the breast epithelial architecture was strongly and significantly associated with disease-specific and overall survival rates. Thus, the use of automated fractal analysis provides a novel approach to developing better objective prognostic indicators for health care professionals.

**Machine Learning**

Pathologists must be able to recognize morphological patterns. This ability depends on training: the more slides pathologists review in their training, the better they become. Through this training, pathologists learn to associate visual patterns with disorders. Pattern-recognition algorithms operate on a similar scheme, with recent advances showing that combining unsupervised (implicit) and supervised (explicit) learning can train algorithms to learn statistical regularities for nearly any data. Therefore, the application of machine learning to digital pathology is a natural fit and offers potential for advancing the field.

The Ki67 proliferation index is a valuable tool for evaluating the progression of neuroendocrine tumors and predicting therapeutic response. Modalities to quantify the Ki67 proliferation index, the ratio of the number of immunopositive tumor cells to all tumor cells, are subjective and imprecise. To create an objective and reproducible quantification process, Xing et al. developed an automatic Ki67 counting method. Their algorithm creates a “dictionary” of visual features of cells and then uses those features to identify cells in images and subsequently count them with high precision. Their method performs comparably to the manual count of the pathologist, beating out any existing method that utilizes machine learning.

Mitotic counts have been a staple in the field of pathology for determining the prognosis of different types of cancers. However, these counts have many of the same disadvantages as the aforementioned Ki67 proliferation index. Ciresan et al. implemented a deep neural network to detect mitosis in slides stained with hematoxylin and eosin from samples gathered from patients with breast cancer. The deep neural network operates in a feedforward manner, with simple pixel patterns becoming successively more advanced representations as they pass through the network. This method was powerful for detecting mitotic figures and outperformed all competing approaches examined by Ciresan et al.

**Circulating Tumor Cells**

Circulating tumors cells (CTC) are an ever-expanding field of personalized medicine and are the front line of cancer treatment. CTCs are released into the blood via malignant tumors and can be detected in the blood with a highly sensitive and specific capture method. Current techniques face the challenge of detecting the minute numbers of CTCs among the plethora of white and red blood cells circulating in the blood. A novel application related to CTCs for digital pathology might be in the detection of these CTCs. Through the use of immunofluorescence-staining protocols, various CTC subtypes can be identified through sophisticated algorithms written to interpret stained results. This would create an objective and reproducible method for subtyping and quantifying the CTCs.

For more information about CTCs, please review the article written by Cáceres and colleagues in this issue of Cancer Control.

**Conclusions**

In the early era of personalized medicine, digital pathology will continue to advance, provide precise prognostic information, and help guide treatment decisions. Digital image analysis is likely to improve the assessment of predictive biomarkers in tissue sections and overcome the challenges associated with subjective analyses. With such a significant interest in immunotherapy, digital image analysis is likely to provide solutions to better assess the tissue microenvironment for immune cell components, much like digital analysis for in situ flow cytometry.

The potential of digital pathology is vast. In the field of cytopathology, automated digital analyzers exist for reviewing Papanicolaou staining; however, they are costly and their use is limited, thus making analyzers less cost effective than cytotechnologists. However, with the rapid increases in computing power and concomitant decreases in cost, it may be only a matter of time before such analyzers become more common.

Beck et al. demonstrated that automated morphometric analyses can play a major role in teaching.
medical professionals about important prognostic morphological features, but will such analyses become cost effective enough for routine clinical use? No doubt these tools will continue to further our understanding of disease and improve the abilities of pathologists to make better informed decisions related to prognosis and treatment. In addition, as machine learning advances, so does the field of digital pathology. In the future, software may be able to handle large throughput volume and highly complex, multidimensional data, thus assisting pathologists with reducing diagnostic errors and rendering objective, accurate, and reproducible diagnoses, all of which are likely to improve clinical care and decrease the cost of health care.

References


New technologies for molecular analysis are being routinely used in diagnostics.

Molecular Technologies in the Clinical Diagnostic Laboratory
Victor E. Zota, MD, and Anthony M. Magliocco, MD

**Background:** New technologies for molecular analysis are increasing our ability to diagnose cancer.

**Methods:** Several molecular analysis technologies are reviewed and their use in the clinical laboratory is discussed.

**Results:** Select key technologies, including polymerase chain reaction and next-generation sequencing, are helping transform our ability to analyze cancer specimens. As these technological advances become more and more incorporated into routine diagnostic testing, our classification systems are likely to be impacted and our approach to treatment transformed. The routine use of such technology also brings challenges for analysis and reimbursement.

**Conclusion:** These advances in technology will change the way we diagnose, monitor, and treat patients with cancer.

**Introduction**
Molecular analysis is transforming the way health care professionals approach cancer. Fundamentally, cancer is primarily a disease of DNA. Driver mutations drive clonal proliferation. Some cancers acquire disrupted genomes, often as a consequence of defective DNA repair processes that may be either inherited or acquired. Some of these driver mutations present attractive targets for therapy, such as mutant EGFR and BRAF, whereas others may predict a more aggressive course of cancer, such as mutations in p53. The classification of cancer is trending away from histomorphological determinants and toward functional and molecular subcategories. Insights gained from deep sequencing helps us understand tumor evolution and the emergence of new subclones that may be resistant to therapy. Applying methods of molecular analysis to cancer treatment is likely to become routine practice, and the information derived from such analysis can then help guide classification and treatment selection. Furthermore, advances in trace DNA measurements will allow the molecular monitoring of patients and the effective analysis of tumor progression, thus enabling the creation and application of more adaptive therapies for patients.

**Polymerase Chain Reaction**
Polymerase chain reaction (PCR) has revolutionized molecular biology. This single advance enabled the trace amounts of DNA to be amplified so as to enable other techniques, such as sequencing, to be performed. The technology was based on the discovery of a thermally stable polymerase enzyme taq isolated from *Thermus aquaticus*, which can live at temperatures approaching the boiling point in hot streams.¹

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The key aspect of this thermally stable enzyme is that it survives repeated episodes of heating and cooling and can still carry out DNA polymerase activity. PCR is based on sequentially and exponentially denaturing double-stranded DNA and synthesizing new strands in target areas defined by “primers” to exponentially amplify areas of interest in each round. Typically, 20 to 30 rounds of DNA amplification cycles are performed that enable the massive amplification of a target. Specific areas for targeted amplification are defined by the primer sequence. Consequently, the target and flanking sequences must be known and the primers are artificially synthesized.

PCR has numerous key applications, including identifying rare sequences (e.g., presence of viral DNA in a tissue sample) or amplifying a target gene so that a sufficient quantity can be applied for standard sequencing methods. PCR has improved following the introduction of real-time or quantitative PCR (qPCR), which incorporates a probe that either emits or quenches a signal after each round of PCR. This modification enables the direct monitoring of the progression of PCR, typically by photoelectrical means to precisely determine the quantity of the starting target and product. This method is valuable for quantitatively determining targets such as the number of viruses or the mutational load in a biosample.

**Digital Polymerase Chain Reaction**

In 1999, Volgelstein and Kinzler introduced the term “digital PCR,” which is a modification of the original PCR method in which the reactions are partitioned into subcompartments and the reaction is carried to completion. The goal is to dilute the specimen to the point where 1 target is present in each compartment, thus enabling precise, single-target quantification with either a positive or negative result for each partition (hence the term “digital”). This analytical method is based on the principles of the Poisson distribution. Oftentimes, the compartmentalization is accomplished by creating an emulsion. This method has improved accuracy rates for sensitivity and specificity of the reaction and is capable of detecting rare targets.

Droplet-digital PCR is qPCR in a droplet. In each droplet is a mini-PCR reactor that contains a single DNA molecule, primers, Mg ions, molecules, dinucleotides, a polymerase molecule, and a fluorescently labeled, allele-specific detection probe. qPCR functions on the same principle as PCR, but a fluorescently labeled detection probe is added to the process. The probe incorporates a 5’ reporter dye and a 3’ nonfluorescent quencher and is designed to be complementary to a specific allele (wild-type or mutant). During the amplification process, DNA polymerase removes the quencher from the probe, thus unmasking the fluorescent signal. A single DNA molecule in a droplet is then amplified by PCR, fluorescently labeled, and read via automated droplet flow cytometry. Each droplet is assigned a positive or negative value based on the rate of fluorescent intensity. The number of positive and negative droplets is read via flow cytometry and is used to calculate the concentration of an allele.

An advantage of digital PCR is low DNA template requirements. In the era of personalized medicine, this fact is of particular importance because laboratories are provided with minimal amounts of tissue triaged for diagnostic purposes and molecular characterization. In addition, digital PCR typically does not require a preamplification step for increasing the abundance of all sequences of interest and does not alter the relative abundances of these sequences. However, unbiased amplification is difficult to achieve; thus, PCR biases are sometimes introduced.

**Clinical Application:** Digital PCR can be used for any testable DNA sequence that can be detected with standard PCR; digital PCR also has the advantage of target sequence quantification, which can be used to detect rare variants in a population of DNA molecules and to estimate the frequency of a variant sequence or the relative copy number of separate sequences in template DNA.

Digital PCR is likely to have widespread use for screening and the monitoring of minimal residual disease. Its limitation is that a specific mutation or target sequence must be known in advance, and a digital PCR assay must be designed for this specific requirement.

**Detecting Clinically Significant Rare Variants**

The genetic makeup of tumors is heterogeneous, meaning that tumors are composed of various cell populations with distinct mutations. Although most somatic mutations are present in all tumor cells, additional mutations are acquired with every tumor cell division and, if that mutation confers a survival advantage, then distinct subclones will evolve. The ability to detect the presence of targeted therapy-resistant subclones in a tumor could alter our approach to treatment. It may be possible to accurately predict which tumors are intrinsically resistant to therapy prior to treatment commencement. It may also be possible to identify tumors at risk for developing resistance during the course of treatment.

Several known key “actionable” mutations are linked to therapies approved by the US Food and Drug Administration (FDA). These include mutations of EGFR, PIK3CA, and KRAS. Digital PCR may help detect these key actionable mutations in biofluid specimens such as plasma or urine, leading to a more rapid diagnosis and initiation of treatment when tissue is lacking; in addition, the use of digital PCR may also help us accurately monitor treatment response.
through the quantitative assessment of mutational load in the biofluid.5,12-15

With its sensitivity rate, digital PCR enables the tracking of individual tumor clones that may result in tailoring therapies to delay disease recurrence. Within the era of personalized medicine, it is insufficient to detect mutant alleles alone. Instead, one should quantify the allele burden to suggest the clinically relevant threshold at which targeted therapies are the most effective and subsequently modify therapies to avoid or delay the emergence of resistance. One example of this approach is the quantification of KRAS-mutant alleles in metastatic colorectal cancer. KRAS mutations are predictive of nonresponse to anti–epidermal growth factor receptor (EGFR) therapies in metastatic colorectal cancer, but 50% of patients without the mutations benefit from these therapies.16 When using digital PCR for the quantification of the mutant KRAS allele, a reverse correlation was seen between the proportion of mutated DNA and the frequency of anti-EGFR response ($P < .001$). In addition, patients with fewer than 1% of mutant KRAS alleles have similar progression-free and overall survival rates than those with wild-type KRAS tumors.16

Evidence is encouraging that the supreme sensitivity of digital PCR could be exploited as a screening tool to detect cancer in earlier stages. For example, the detection of KRAS, APC, and TP53 mutations in fecal, urine, or blood samples may be a strong indicator of an underlying colonic malignancy.5,6,7,17,18

Digital PCR can also be used to estimate copy number variation (CNV), which is a known mechanism employed by cancer cells to alter gene expression.19

Digital PCR is expected to revolutionize the monitoring of minimal residual disease for diseases with a key molecular indicator, such as the BCR-ABL fusion in chronic myelogenous leukemia.20 In addition, the technology may be able to monitor the emergence of resistance clones to key targeted therapies, such as EGFR T790M mutations in non–small-cell lung cancer, which may predate the emergence of therapy-resistant metastasis.18,21,22 These applications form the basis of the “liquid biopsy,” enabling the real-time monitoring of the evolution and progression of the tumor as well as the emergence of therapy resistance. Access to real-time data may lead to the consideration of adaptive treatment designs and the more rapid discontinuation of potentially toxic and medically futile treatments.21

Researchers have demonstrated that detecting a mutant allele at a ratio of 1:100,000 is possible when using a microdroplet system.5,23 This technological advance may lead to opportunities to study minimal residual disease and real-time responses to therapeutic interventions.

**Sanger Sequencing**

Molecular pathology owes its existence to our ability to sequence DNA. Specific sequence information is a prerequisite for designing the primers and probes used in diagnostic tests. The DNA sequencing method originally developed by Sanger et al24 is the basis for most DNA sequencing performed in clinical laboratories. It is also the backbone technology that enabled the sequencing of the human genome.

Sanger et al24 used the principles of DNA replication to develop the process now known as Sanger dideoxy sequencing, which is a method based on a combination of DNA polymerase reaction primed with specific sequencing primers, along with either radioactive nucleotide or dye termination. The products of the reaction are electrophoretically analyzed, which enables a direct sequence to be determined.25 Detection software generates an electropherogram of the DNA sequence, correlating each rate of fluorescence intensity of each dye to a specific dideoxynucleotide triphosphate migration time. Capillary electrophoresis facilitated the widespread incorporation of sequencing and fragment analysis into the clinical laboratory.

Pyrosequencing is a method that allows the rapid sequence determination of short DNA fragments. The reaction is based on detecting the release of the pyrophosphate (PPi) byproduct after the nucleotide is incorporated into the growing DNA chain. Several enzymes, as well as DNA polymerase, are required, including adenosine triphosphate (ATP) sulfurylase, luciferase, and apyrase. After primer hybridization, deoxynucleotide triphosphates (dNTPs) are sequentially added to the growing chain by DNA polymerase, and this process results in the release of PPi. Because dNTPs are individually added to the reaction, their incorporation can be determined based on the presence or absence of PPi. Therefore, for each dNTP incorporated, an equimolar quantity of PPi is detected. The enzyme apyrase is used to degrade unincorporated dNTPs and excess ATP prior to the next dNTP. Detection is based on the conversion of PPi to ATP by the enzyme ATP sulfurylase. The resulting ATP provides the energy required for luciferase to convert Luciferin to oxyluciferin, thus generating visible light, which is detected using a charge-coupled device camera. The sequence is then displayed.

**Mass Spectrometry for DNA Mutational Analysis**

The MassARRAY System (Sequenom, San Diego, California) capitalizes on the previous work of many scientists.26,27 In 1988, Karas and Hillenkamp28 first reported the use of an organic molecule as a matrix to assist desorption/ionization of other small molecules under ultraviolet laser irradiation. At the same time, Tanaka et al29 showed that analytes can be ion-
ized using a mixture of metal powder and glycerol. This method became known as soft ionization. This advance enabled the technique to be applied to larger biomolecules. Karas et al. devised a method of matrix-assisted laser desorption/ionization (MALDI), and, subsequently, Tanaka et al. showed that coupling MALDI to a time-of-flight (TOF) mass analyzer allowed the detection of macromolecules (in particular, proteins). This method of MALDI-TOF mass spectrometry (MS) for the analysis of macromolecules created new opportunities for applying MS to biomedical research.

In MALDI-MS, a sample is embedded in the crystalline matrix and deposited on a conductive sample support. A nanosecond laser beam then disrupts the sample embedded in a matrix. The resulting ions are then manipulated by an electrical field. The ions are accelerated and sent in a vacuum flight tube where they are separated according to their speed, which is related to the mass:charge ratio. Spectra are then analyzed and the ionic masses are isolated based on TOF. The spectra produced by this method are generally simple and do not require excessive processing for interpretation.

In 1992, Nordhoff et al. first demonstrated the use of MALDI-TOF MS to detect and measure the masses of nucleic acids. The most commonly used method for MALDI-TOF MS is detection and quantification of single-base, primer-extension products for qualitative and quantitative analysis of DNA copies containing single nucleotide polymorphisms by MALDI-TOF MS. This method is applied to a selected region, which is amplified by PCR along with a single-base primer extension reaction, enabling the resolution of 4 different base results. The data are presented in Sequenom proprietary software.

The single-base primer extension assay can be applied to the diagnosis and screening of any pathological conditions known to have mutations in a specific gene or a specific set of genes easily identified and quantified by this method. This assay was used to detect and quantify the frequency of EGFR-activating mutations in the tissue of a patient with non–small-cell lung cancer to predict response to tyrosine kinase inhibitors. This is a powerful method for resequencing projects as it overcomes many of the limitations of gel capillary–based electrophoretic methods because of its resolution. It is also ideal for detecting frameshift or single-base polymorphisms (single nucleotide polymorphisms).

**Somatic Mutation Analysis**

Several multigene panels have also been developed for the MassARRAY System, and these panels survey select key oncogenes for selected mutations. For example, LungCarta (Agena Bioscience, San Diego, California) surveys 26 genes for 214 different mutations. This system is used in many clinical laboratories because of its high sensitivity and quantitative aspects. It is also flexible in the input samples and can analyze DNA derived from frozen tissue, formalin-fixed paraffin-embedded tissue, fresh tissue, and cell lines. It can also efficiently and cost effectively survey numerous known mutation targets in thousands of samples. Data analysis is performed using Typer Analyzer (Sequenom), an analysis software with customizable parameters that can be adjusted on desired probabilities, including mutation frequency.

**Digital Molecular Barcode Technologies**

The NanoString Protocol (NanoString Technologies, Seattle, Washington) quantifies nucleic acid molecules and is frequently applied to the quantification of ribonucleic acid (RNA) expression. The system uses a combination of isolation probes to capture targets of interest coupled with a detection system with a directly observable signal generated by an attached reporter bar code that can be optically observed by a scanner system. This technology directly isolates and quantifies the target molecules without the need for an amplification step. This is an advantage because amplification biases and errors can be avoided and targets can be digitally and precisely counted so that measurements of expression are accurate over several orders of magnitude. The technology utilizes a similar approach to measure messenger RNA expression and DNA CNVs. Up to 800 regions of interest can be studied with 1 probe mix/1 hybridization.

The Prosigna (NanoString Technologies) assay uses a proprietary algorithm to determine risk for breast cancer recurrence using multiplex gene expression determination. The assay is based on the Prediction of Microarrays (PAM50; NanoString Technologies) assay, which uses 50 genes to classify breast cancer into intrinsic subgroups and calculates risk of recurrence at 10 years. It is available in Europe and has received clearance by the FDA. The assay can be used for estrogen receptor–positive breast cancer in postmenopausal women who have fewer than 3 positive axillary lymph nodes. The assay has similar performance characteristics to Oncotype DX (Genomic Health, Redwood City, California), Mammostrat (Clarient, Aliso Viejo, California), and the immunohistochemical 4 score.

For various subtypes of leukemia, the nCounter Leukemia Fusion Gene Expression Assay Kit (NanoString Technologies) detects fusion genes resulting from balanced translocations. In addition, the assay detects 25 fusion genes and the expression of 23 biomarkers, including TP53, RB1, NRAS, EVII, MN1, MLLT11, FLT3, and WT1, among others. The kit can be broadly applied to DNA extracted from select tissues and specimens, including formalin-fixed,
paraffin-embedded samples as well as from blood and bone marrow biopsy specimens. The test is cost effective, less sensitive to the quality of RNA, and less challenging in data analysis, thus making it preferable for the screening of fusion events with known break points as well as for examining CNV.

NanoString Technologies is also working on ways to mark antibodies to enable the capture and quantification of targeted proteins.53

Next-Generation Sequencing
Until recently, molecular diagnostic laboratories relied on low-throughput molecular and cytogenetics methods designed to interrogate the most frequent mutations (hot-spot mutations) in cancer. The methodology limited the number of mutations or target regions tested in an assay. With the growing number of mutations and genes being discovered in a variety of cancers, such as lung, breast, melanoma, and brain cancers, molecular diagnostic laboratories have begun to implement next-generation sequencing (NGS) to interrogate numerous mutations in a timely and cost-effective manner.

Several major NGS technologies are available, and sequencing by synthesis technology from Illumina (San Diego, California) dominates the market. Its NGS technology uses solid-based amplification of small DNA fragments and sequentially identified composing bases detected during synthesis of a complementary DNA strand. Performing NGS is not dissimilar from other sequencing technologies in that the procedure requires the standard steps of nucleic acid isolation, performance of the assay, followed by detailed data analysis.54 The first step is DNA isolation followed by fragmentation and enrichment of the target genes,54 producing a set of short DNA fragments (100–500 base pairs) flanked by oligonucleotide adapters during the library generation process.55 The adapters are complementary to immobilized oligonucleotides on a flow cell, and the resultant libraries are subjected to a clonal amplification step prior to sequencing.56 The proprietary technology utilizes bridge amplification to form template clusters on a flow cell. The company has developed proprietary sequencing by synthesis chemistry in which fragments or “clusters” are sequenced base by base by adding fluorescent-labeled terminator nucleotides optically imaged after each step. The process is repeated until the fragment is analyzed by the required reaction cycles. After the sequencing platform generates the sequencing images, the data are analyzed in 5 steps: (1) image analysis, (2) base calling, (3) Bcl conversion, (4) sequence alignment, and (5) variant analysis and counting.

Another new technology is Ion Torrent (Life Technologies, South San Francisco, California). The Ion Personal Genome Machine (PGM; Life Technologies) uses semiconductor sequencing technology. After the nucleotides are incorporated into the DNA molecules via polymerase, a proton is released, resulting in minute changes in pH recognized by the PGM, which in turn interprets whether a nucleotide has been added or not. The biofluidic semiconductor chip is sequentially flooded with one nucleotide after another, resulting in voltage spikes when incorporation has occurred. If a dinucleotide or polynucleotide repeat is observed, then the voltage spike will be double in the target microscopic well.57,58 Because the PGM does not require fluorescence and camera scanning for signal detection, the instrument is small in size and can sequence at high speeds and at a low cost per base. The major limitation of semiconductor sequencing is a relatively small sequencing length (200 bp) and poor nucleotide resolution in long homopolymeric DNA regions. The Ion Torrent PGM can also be customized with an Ion Ampliseq (Life Technologies) panel of 200 to 300 actionable genes (single nucleotide variant, selected translocation, gene amplifications) and is being used for eligibility testing for the Molecular Analysis for Therapy Choice program of the National Cancer Institute.59

Massively parallel (next-generation) DNA sequencing, combined with declining sequencing costs, as well as increased technical feasibility and maturing to support the bioinformatics infrastructure, is being used in hospital laboratories because the technology cost-effectively evaluates multiple targets for the presence of mutations in a variety of specialties, including oncology, genetics, infectious diseases, and others.60-62 Targeted panels are the most suited for the clinical application of NGS assays and have improved the diagnostic yields of molecular testing. However, for a new assay to become effectively utilized in the health care system, it must have high rates of sensitivity, accuracy, precision, and specificity, and it must demonstrate effective clinical utility.

So, does NGS make a difference in patient care? Tests have been developed that rapidly analyze tumor DNA to detect hundreds of variants that may drive cell growth and to provide clues about treatment options.62 In addition, the sequencing of circulating complementary DNA has detected chromosomal abnormalities associated with tumors, suggesting that the approach may be a viable option for the non-invasive detection of cancer.63 Although NGS technology can effectively identify key driver mutations, such as activating mutations in EGFR in lung cancer, thus serving as a reliable guide for selecting tyrosine kinase inhibitor therapy,62 its use is still early for oncologists as the whole. For example, these advanced sequencing technologies bring new challenges to the molecular diagnostic laboratory, including questions about how to manage complex and large data sets,
validating bioinformatics pipelines, and learning how to interpret the data to make clinically meaningful treatment recommendations.64-66

The College of American Pathologists has recognized these challenges and is working to create proficiency testing for NGS clinical laboratories that focuses on determining laboratory performance in both the “wet” and “dry” laboratory components of NGS clinical analysis.67

Making Sense of the Cancer Genome
As we are becoming technologically proficient and capable of generating complex analyses of genomic changes in cancerous tumors, a major challenge still exists to understand how such changes contribute to tumor biology or behavior.68 We must also learn how to exploit these changes in the tumor to improve treatment strategies with targeted therapy agents. Existing successes in personalized medicine capitalized on the genomic knowledge accumulated from decades of research68, however, to continue on the path of personalized medicine, we must streamline the methods of clinical validation of newly discovered targets.

The scientific and medical communities must develop strategies to manage the torrent of emerging information regarding the genetic underpinnings of cancer and the potentially targetable and actionable mutations now routinely detected in the hospital laboratory. This has created a situation in which our knowledge of the biological makeup of cancer is far ahead of high-quality evidence about how to use such knowledge to select therapy. Numerous efforts are ongoing to collect the key evidence linking clinical behavior to detectable genetic biomarkers observed in cancers.69,70

Foundation Medicine realized the utility of NGS as a clinical tool and in understanding the basic mechanisms of the evolution of cancer. It brought to market Foundation One (Foundation Medicine, Cambridge, Massachusetts), a Clinical Laboratory Improvement Amendments–validated NGS for solid tumors. This test is a comprehensive genomic profile of 315 genes linked to the pathogenesis of solid tumors.71 The company also developed FoundationOne Heme (Foundation Medicine) for hematological malignances and sarcomas. It is an expanded panel of 405 genes and translocations at 265 loci.72 Sarcomas and hematological malignances are known for the presence of gene fusions that result in the formation of oncogenes that are diagnostic, prognostic, and predictive.72 Thus, the results of both tests can be used to inform the healthcare team about clinically actionable mutations and to help guide treatment options for patients.

However, mutations are frequently detected that suggest a possible targeted therapy for a specific patient, but specific evidence may be missing that the therapy might have a beneficial effect on the disease in that particular individual, thus creating significant challenges in how to apply these results. Ideally, patients with newly identified mutations would agree to participate in “basket” or “match” trials to ensure that the effects of treatment are recorded so as to increase our knowledge base for future patients.59,75

Tumor Heterogeneity
In addition to addressing tissue requirements, NGS technology helps understand the phenomenon of tumor heterogeneity. Intratumoral heterogeneity refers to biological differences between malignant cells originating within the same tumor. During tumorigenesis, the cells acquire necessary driver mutations and many somatic genetic alterations that do not seem to confer selective advantage (passengers). During tumor expansion, unstable tumor genomes give rise to substantial diversified tumor cell populations.73 Intratumoral heterogeneity in driver mutations and in loci implicated in therapeutic resistance leads to clinical heterogeneity in tumor response to treatment.74-77 A challenge facing us is the identification of these driver mutations for diagnostic purposes and targeted therapy development. This is complicated by the fact that classifying mutations into passengers and drivers is context-specific and, as tumors change over time, the selective value of a given mutation changes.75 In addition, mutations in driver genes are often different, thus altering different codons. For example, the KRAS G12D and KRAS G13D mutations do not appear to have the same clinical implications; in fact, most activating mutations may have different effects on cancer cells.9,48,78,79

Genetic heterogeneity provides the substrate for fueling tumor evolution during tumor progression and therapeutic resistance.73 Target therapy based on founder mutations may eradicate the primary tumor, but most patients with complete responses invariably relapse. One example of this is the activating mutation of BCR-ABL in chronic myelogenous leukemias and KIT in gastrointestinal stromal tumors, which tend to develop additional mutations, leading to loss of the ability of the drug to bind to its target.80,81

Tumors may also escape targeted therapy by activating survival pathways such as those observed in BRAF V600E–mutant melanomas treated with vemurafenib.82 Resistance often develops via activation of the survival pathway via the receptor tyrosine kinase or the RAS-mediated reactivation of the mitogen-activated protein kinase pathway.83 In lung cancer treated with EGFR inhibitors, resistance is developed by activation of alternative prosurvival signaling pathways via MET amplification, as well as for accumulation of the EGFR T790M mutation, which affects the binding of tyrosine kinase inhibitors.74,84

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Cytotoxic therapies such as cisplatinum applied to cancer may also result in the selective destruction of sensitive clones and the selection of resistant clones, thus driving tumor evolution. Therefore, the genetic heterogeneity of a tumor is an important determinant of therapeutic outcome that requires comprehensive tumor characterization before, during, and after the initiation of therapy.

**Classification**

Historically, disease classification schemes in pathology were based on the histological and morphological appearance of the neoplasm and the tissue of origin. Advanced molecular analysis technologies have created the opportunity to augment traditional classification systems with molecular features, such as the nature of the driver mutation (e.g., presence of mutant *BRAF* in malignant melanoma) that might associate a specific subtype of disease with a particular etiology, risk factor, behavior, or treatment option.

Similarly, molecular classification using messenger RNA expression patterns have altered the way breast cancer is evaluated, resulting in a molecular subdivision of classification that defines new molecular and biological subtypes, including luminal types A and B, human epidermal growth factor receptor 2, and basal subtypes. Recognition of these types has led to the creation of new diagnostics and has altered the way clinical trials are considered and designed. The development of trials focusing on molecular features, rather than tissues of origin, are highlighted by “basket” and “match” designs that enroll participants based on the presence of specific mutational driver mutations. However, caution should be exercised because some cancers with activating driver mutations may not equivalently respond to a targeted treatment. An example of this is the use of v-raf murine sarcoma viral oncogene homolog B (BRAF) inhibitors in colorectal cancer displaying activating mutations of *BRAF*. Although BRAF inhibitors appear to inhibit melanomas with *BRAF*-activating mutations, disappointing results were seen in study volunteers with *BRAF*-mutated colorectal cancer. This failure has been attributed to the bypass of the BRAF-signaling system by the EGFR-signaling pathway in colorectal cancer, a pathway not active in melanoma. Consequently, future approaches may rely on “basket” or “match” designs in which a large set of therapeutic options can be utilized against a range of underlying driver molecular targets.

**Clinical Implementation**

The development and rapid widespread adoption of low-cost NGS into the clinical laboratory have changed the way we think about cancer. The technology has also challenged pathologists and other health care professionals to carefully think about how to interpret data and incorporate them into meaningful treatment planning. The main challenge facing many laboratories is how to validate the analytical and clinical performance of these advanced tests. NGS technology also poses major challenges for regulatory agencies. The rapid adoption of these technologies is outpacing the abilities of governmental agencies to develop effective monitoring strategies. Cancers are dynamic, heterogeneous entities that harbor a diverse range of genetic alterations that drive tumor progression and passenger mutations. In addition, tumors evolve biological subclones that possess various phenotypes over time and in response to therapy. The diversity of this biology is fundamentally challenging the way we gather evidence, because it makes classical clinical trial design difficult, if not impossible (e.g., conducting a randomized blinded trial for each mutation is not feasible). Consequently, future approaches may place greater emphasis on “basket” or “match” designs in which a large set of therapeutic options can be utilized against a range of underlying molecular targets.

**Investment Uncertainties**

The rapid adoption of personalized medicine testing has also been hampered by the reimbursement landscape. For example, Current Procedural Terminology codes identify surgical, medical, and diagnostic services used by insurance companies for reimbursement of services rendered. Genetic sequencing technology is advancing faster than it can be incorporated into the health care delivery system, and this is evidenced by the lack of specific Current Procedural Terminology codes for genetic testing.

To recoup the capital investment in equipment and procedural development, some laboratories use a
billowing technique known as code “stacking” to recover costs. The problem with a cost model for reimbursement is that no premium is placed on innovation or clinical utility as would be apparent in a value-driven system, a model used by the pharmaceutical industry to set drug prices. Reimbursement of diagnostics is set on a case-by-case basis by contractors of the Centers for Medicare & Medicaid Services. In addition, some decisions regarding pricing have resulted in fees set at prices so inadequate that laboratories cannot cover the cost of such testing, a fact that has led to the bankruptcy and closure of some molecular laboratories in the United States.

It is clear that, in this era of advancing technology and novel treatment options, molecular pathologists and oncologists must closely work with patients and payers to develop a strategy to enable a sustainable provision of molecular testing technology, along with a system to reward innovation and assess proper value to these analytical technologies.

Conclusions
We are living in a revolutionary time in the field of diagnostic pathology. The rapid introduction of next-generation sequencing and other high-complexity testing technologies are altering our understanding of cancer and our capability for detecting, monitoring, and selecting treatment. This updated knowledge is likely to impact our classification systems and transform our approach to cancer treatment. Thus, it is vital that we learn how to effectively and sustainably deploy and interpret the data from these new technologies to produce the most benefit for patients with cancer.

References


Molecular markers refine
the cytopathological diagnosis
of thyroid neoplasms.

Molecular Assays in Cytopathology for Thyroid Cancer
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Background: Despite lack of adequate, validated, independently performed clinical studies, several molecular tests are commercially available on the market and are being used on indeterminate thyroid nodules to guide patient-care decisions.

Methods: We summarize the current evidence on the role and limitations of molecular tests used in combination with thyroid cytopathology to refine the presurgical diagnosis of thyroid nodules.

Results: The clinical performance of molecular tests depends on the pretest risk of malignancy within the specific cytological group being assessed. This risk is variable and should be assessed at each institution to optimize the selection of the molecular test and the interpretation of its results. Next-generation sequencing has increased the sensitivity of oncogene panels while maintaining high specificity. Tests assessing the gene expression pattern have shown promising results, with high sensitivity but low specificity. The impacts of molecular markers on clinical practice remains in flux and their effect on health care costs remains poorly understood.

Conclusions: Further large, independent, confirmatory, clinical validation studies and real-world, cost-effectiveness studies are necessary before the widespread adoption of these tests can be endorsed as standard of care.

Introduction
The incidences of benign thyroid nodules and thyroid cancer have increased in the last several decades. An accurate presurgical diagnosis of thyroid nodules is important because of the implications in clinical management. Although fine needle aspiration (FNA) cytology provides valuable information in the presurgical evaluation of thyroid nodules, approximately 25% of biopsy samples do not render diagnostic information and are classified as indeterminate.

Repeating FNA on an indeterminate specimen may be helpful at times; however, for many patients, diagnostic surgery is eventually needed to clarify the diagnosis. One-third of these nodules resected for diagnostic purposes will prove to be malignant and, in many cases, will require additional surgery to complete thyroidectomy. Conversely, two-thirds of such surgeries might have been avoided with a more accurate presurgical diagnosis.

The Bethesda System for Reporting Thyroid Cytopathology (Bethesda) has standardized reporting terminology and diagnostic criteria, but it has not improved the performance of FNA. Indeterminate specimens are stratified by Bethesda in 3 diagnostic
categories according to risk of malignancy (ROM):

- Category III: Atypia/follicular lesion of undetermined significance
- Category IV: Follicular neoplasm/suspicious for follicular neoplasm and Hürthle cell neoplasm/suspicious for Hürthle cell neoplasm
- Category V: Suspicious for malignancy

The estimated ROMs of Bethesda categories III to V are 5% to 15%, 15% to 30%, and 60% to 75%, respectively. However, the observed ROMs in the indeterminate categories, particularly Bethesda categories III and IV, are broader, ranging from 0% to 48% and 14% to 49%, respectively, in multiple large institutional studies.

This diagnostic challenge reflects subtle morphological differences and significant morphological overlap in the cytological and histological features exhibited by thyroid follicular pattern lesions, including follicular hyperplasia, adenomatous nodule/follicular thyroid adenoma, follicular thyroid carcinoma, and the follicular variant of papillary thyroid carcinoma (PTC).

The subjectivity of the pathological diagnosis further complicates this issue. Broad intraobserver and interobserver variabilities have been reported for follicular thyroid lesions in cytological and histological specimens. Several molecular tests have been developed in an attempt to better and more reliably characterize these lesions to avoid diagnostic surgery. These tests use different approaches to identify molecular signatures specific for thyroid cancer. Some scrutinize the DNA to detect mutations and others characterize the gene expression profiles at the transcriptional (messenger ribonucleic acids [RNAs]) or post-transcriptional level (microRNAs). All these methods have limitations and no single test is 100% accurate. Furthermore, the predictive values of these tests — which represent their true clinical value — depend on the cytological ROM, which represents the pretest probability of malignancy, as the ROM increases, so does the positive predictive value (PPV) at the expense of a reduction in the negative predictive value (NPV), and vice versa. Therefore, until further standardization is achieved in the classification of indeterminate cytology, the results obtained at one center may not extrapolate to others. Marketing a test on the basis of a fixed NPV, without first knowing the true pretest ROM, is disingenuous.

### Table. Common Mutations and Rearrangements in Common Thyroid Tumors

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Mutation and Rearrangement</th>
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<tbody>
<tr>
<td>Papillary thyroid carcinoma</td>
<td>BRAF V600E (classic, tall cell variant)</td>
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<tr>
<td></td>
<td>TERT</td>
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<tr>
<td></td>
<td>RET/PTC1 (RET/CCDC6)</td>
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<td></td>
<td>RET/PTC3 (RET/ELE1)</td>
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<td></td>
<td>RET/PTC6 (RET/HTF1)</td>
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<tr>
<td>Papillary thyroid carcinoma (follicular variant)</td>
<td>EIF1AX</td>
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<tr>
<td></td>
<td>BRAF K601E</td>
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<tr>
<td></td>
<td>NRAS</td>
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<td>HRAS</td>
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<td>KRAS</td>
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<tr>
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<td>PAX8/PPARγ</td>
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<tr>
<td>Follicular thyroid carcinoma</td>
<td>PAX8/PPARγ</td>
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<td></td>
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<td>BRAF K601E</td>
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<tr>
<td></td>
<td>TSHR</td>
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<tr>
<td>Follicular thyroid carcinoma (oncocytic variant)/Hürthle cell carcinoma</td>
<td>TP53</td>
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<tr>
<td></td>
<td>HRAS</td>
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<td></td>
<td>KRAS</td>
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<td></td>
<td>PTEN</td>
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<tr>
<td>Poorly differentiated thyroid carcinoma</td>
<td>NRAS</td>
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<td>PIK3CA</td>
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<td>GNAS</td>
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<td></td>
<td>RAF V600E</td>
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<tr>
<td>Undifferentiated thyroid carcinoma (anaplastic carcinoma)</td>
<td>TP53</td>
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<tr>
<td></td>
<td>BRAF V600E</td>
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<td>NRAS</td>
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<td>PIK3CA</td>
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<td>CTNNB1</td>
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<tr>
<td>Medullary thyroid carcinoma</td>
<td>RET</td>
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<td>HRAS</td>
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<td>KRAS</td>
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<tr>
<td>Follicular thyroid adenoma</td>
<td>PAX8/PPARγ</td>
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<td>NRAS</td>
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<td>KRAS</td>
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<tr>
<td>Benign thyroid “hyperplastic nodule”</td>
<td>TSHR</td>
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<td>GNAS</td>
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Data from references 16 to 21.

During recent years, our understanding and knowledge regarding driver mutations have been expanding. A recent publication by The Cancer Genome Atlas (TCGA) has significantly reduced the proportion of PTCs with unknown driver mutations from 25% to 3.5%. Such mutations are almost always mutually

### Somatic Alterations

Most thyroid cancers exhibit somatic point mutations or rearrangements that activate the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 kinase/protein kinase B pathways (Table).
exclusive and homogeneously present within the tumor.\textsuperscript{17,18} Most well-differentiated thyroid cancers exhibit a single point mutation or chromosomal rearrangement, although 2 or more mutations may be found in more aggressive tumors.\textsuperscript{17} Some mutations are highly specific for thyroid cancer such as the point mutation \textit{BRAF} V600E; other mutations, like \textit{RAS} mutations, are also found in benign lesions, thus limiting their diagnostic specificity. However, this finding could be explained by the evolution hypothesis, which posits that the accumulation of mutations induces progression from a polyclonal proliferation (hyperplasia) to a benign monoclonal proliferation (follicular thyroid adenoma) to a differentiated thyroid cancer (PTC [follicular variant] or follicular thyroid carcinoma) and potentially from there to undifferentiated thyroid cancer. If this concept holds true, then a \textit{RAS} mutation present in follicular thyroid adenoma would represent an early neoplastic change that might progress to malignancy (precancer) rather than represent a false-positive result.

The presurgical identification of the oncogenic driver mutation in thyroid cancer has been explored for several years as a method to segregate thyroid nodules with indeterminate cytology. Initial studies searched for 1 or just a few specific mutations.\textsuperscript{23-28} If researchers must focus on a single marker, then the most useful mutation is \textit{BRAF} V600E for several reasons: (1) It is the most prevalent mutation among PTC (45%) and PTC represents 85% of all thyroid malignancies, (2) its specificity for malignancy is nearly 100%, and (3) it can independently predict extrathyroidal extension and lymph node involvement, thus implying a higher risk for disease persistence or recurrence that could influence the extent of the surgery.\textsuperscript{29-31} However, the sensitivity of \textit{BRAF} for detecting cancer improves when other frequently occurring mutations are included in the screening panel. Such an observation led to the development of oncogene panels that simultaneously studied several mutations.

The most well-studied panel assessed 14 point mutations in \textit{BRAF} and \textit{RAS} and for \textit{RET}/\textit{PTC1}, \textit{RET}/\textit{PTC3}, and \textit{PAX8/PPARγ} rearrangements. This panel has been prospectively studied in the characterization of indeterminate thyroid nodules achieving estimated sensitivity and specificity for malignancy around 60% and 98%, respectively.\textsuperscript{32-35} Results from the commercially available variant of this panel marketed as mIRInform (Asuragen, Austin, Texas) were published in a validation study that indicated the panel had a slightly worse performance than anticipated, perhaps in part because of differences in the technology used in the commercial version of the test.\textsuperscript{36} In the study, the panel achieved sensitivity and specificity of 48% (29%–68%) and 89% (72%–98%), respectively, for indeterminate thyroid nodules.\textsuperscript{36} A modified version of this test using next-genera-

tion sequencing is now commercially available as ThyGenX (Interpace Diagnostics, Parsippany, New Jersey), but the performance of this modified assay has not yet been reported.\textsuperscript{37}

The advent of next-generation sequencing has allowed the rapid translation of new data on additional, low-prevalence, somatic alterations into the clinical field. An additional commercially available panel is based on this technology and is marketed as ThyroSeq V2 (University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania). This panel now includes more than 400 point mutations in 14 genes,\textsuperscript{38} including \textit{AKT1}, \textit{BRAF}, \textit{CTNNB1}, \textit{GNAS}, \textit{HRAS}, \textit{KRAS}, \textit{NRAS}, \textit{PIK3CA}, \textit{PTEN}, \textit{RET}, \textit{TP53}, \textit{TSHR}, \textit{TERT}, and \textit{EIF1AX}, along with 42 gene rearrangements and can be performed on as little as 10 ng of DNA.\textsuperscript{39-41} In the initial publication on this panel, the test included mutations in 13 genes (rather than the 14 genes now in the commercial offering) and 42 gene rearrangements, and it achieved sensitivity and specificity of 90% (80%–99%) and 93% (88%–98%), respectively, among cytological specimens classified as Bethesda category IV.\textsuperscript{19} Such performance should improve and simplify the interpretation (NPVs and PPVs) of the results among different institutions with distinct pretest ROM. The NPV and PPV achieved in this study were 96% and 83%, respectively, with an overall ROM of 27%.\textsuperscript{19} Based on the reported sensitivity and specificity, we calculate that the NPV will remain above 95% at any ROM up to 34%, whereas the PPV will remain above 75% at any ROM above 15%. As more mutations are added to this panel, we anticipate an increase in the test sensitivity, but it is possible that the specificity could decrease, thus negatively impacting the predictive values. Consequently, although these early results are promising, they still require external validation and additional information regarding the performance of the test on Bethesda category III. Data on this category were presented at the annual meeting and exposition of the Endocrine Society in early 2015, suggesting that sensitivity and specificity are well preserved in this cytological group.\textsuperscript{40}

\textbf{Gene Expression Analysis}

Somatic mutations directly impact the gene expression profile by acting on signaling pathways. For example, \textit{BRAF} V600E is associated with high MAPK signaling, whereas \textit{RAS} mutants have lower MAPK signaling. This observation was used by TCGA to develop a score that quantified the extent to which the gene expression profile of each somatic mutation resembled either \textit{BRAF} V600E–mutant or \textit{RAS}-mutant PTCs.\textsuperscript{17} This score is associated with the degree of thyroid differentiation of the tumor, histological grade, American Thyroid Association risk of recurrence stage,\textsuperscript{3} and the MACIS score.\textsuperscript{41} \textit{RAS}-like tumors were better differentiated and
BRAF V600E–like tumors were less differentiated; in addition, RAS-mutant tumors were homogeneous in terms of gene expression and degree of thyroid differentiation. Conversely, BRAF V600E–mutant tumors had a heterogeneous gene expression profile and included at least 4 different groups, a finding that could explain the uncertainty regarding the prognostic and predictive power of this mutation alone. In light of these results, the authors suggested that PTCs may be more appropriately classified according to their genetic profiles rather than to their morphological appearance. Although these data have the potential to transform histological classification and clinical practice, their utility in routine clinical practice has yet to be established. However, prior studies have already used gene expression profiles (at the transcriptional or post-transcriptional level) to differentiate thyroid nodules with indeterminate cytology; these are discussed below.

Transcriptional Level
Several tools exploit the analysis of the gene expression profile at the transcriptional level to differentiate benign from malignant lesions among indeterminate thyroid nodules. Although several RNA-based markers have investigated single genes, a commercially available multigene expression panel has achieved promising results. The Afirma Gene Expression Classifier (Veracyte, South San Francisco, California) assesses the relative mRNA expression of 167 genes processed through a support vector machine trained on a group of histological and FNA material derived from a broad spectrum of samples from benign and malignant thyroid disease. Using a proprietary algorithm, the classifier reports the nodule as benign or suspicious. In a prospective, double-blind, multicenter study, 265 indeterminate thyroid nodules were analyzed. The overall performance of the test showed sensitivity and specificity of 92% (84%–97%) and 52% (44%–59%), respectively. In this study, the test achieved an NPV of approximately 95% for Bethesda categories III and IV. Such an NPV justifies patient observation in lieu of surgery per the recommendations of the National Comprehensive Cancer Network. However, an NPV of 95% can be assumed only if the midpoint sensitivity and specificity achieved in this single study are used and the ROM of the cytology is below 24%. Because the ROM in the indeterminate categories is higher in many institutions, its use should be individualized according to the specific ROM of each center. In an independent study, the sensitivity and specificity rates achieved by Afirma were reported to be 83% and 36%, respectively, and were the best rates of the case scenarios. If lower sensitivity and specificity than anticipated are confirmed, then both predictive values would be negatively affected. Therefore, further validation is needed before the widespread use of this test.

As single-gene markers, HMGA2 and cancer-related E2 ubiquitin-conjugating enzyme have also been studied in the cytology specimens of indeterminate thyroid nodules. However, neither has demonstrated sufficiently high sensitivity or specificity to justify widespread use. Nonetheless, their combined use, along with other markers, continues to be explored. The detection of thyrotropin receptor mRNA in peripheral blood achieved better results in a study on thyroid nodules with cytological diagnosis of follicular neoplasm, reaching sensitivity and specificity of 97% and 88%, respectively, for detecting thyroid cancer. However, further studies are necessary to validate these results.

Post-Transcriptional Level
MicroRNAs are small, noncoding RNA molecules that bind to mRNA to promote or silence their translation into proteins. These molecules can be analyzed in cytological specimens and those affecting oncogenes or tumor suppressor genes are also being studied. Several studies have reported attempts to identify the specific signatures of thyroid cancer by developing predictive models on excised specimens later investigated as single microRNAs or in microRNA panels on FNA samples of thyroid nodules. One meta-analysis found that panels had better performance rates compared with single microRNAs. The overall sensitivity and specificity of 4 studies using microRNA panels in FNA specimens were 87% (77%–92%) and 85% (78%–90%), respectively. Performance was best in a study validating a panel of 4 microRNAs (222, 328, 197, and 21) in which sensitivity and specificity of 100% and 86%, respectively, were achieved. Larger prospective studies of indeterminate thyroid nodules must be performed to validate their diagnostic utility.

Some microRNAs also appear to have prognostic value. In the study by TCGA, 4 different expression patterns stratified the BRAF-like PTCs into groups with various histological grades of differentiation. The overexpression of microRNA-21 and microRNA-146b, as well as the down-regulation of microRNA-204 — which acts as a tumor suppressor — was found in the group with the most aggressive PTCs. Conversely, higher expressions of microRNA-182 and microRNA-183 were associated with RAS-like PTCs that had a higher degree of thyroid differentiation.

Follicular Neoplasm (Oncocytic Variant)/Hürthle Cell Neoplasm
Most of the molecular markers previously discussed are not helpful for Hürthle cell neoplasms. Hürthle...
cell carcinomas rarely exhibit one of the commonly studied mutations. However, the use of next-generation sequencing might improve the sensitivity of previous oncopanel panels. The initial version of ThyroSeq detected a mutation in 39% of cases of Hürthle cell carcinoma analyzed. Most of them had a TP53 mutation, typically detected in undifferentiated thyroid cancer but not in well-differentiated carcinomas. Moreover, Hürthle cell neoplasms have a distinct profile of genes and transcription factors involved in tumorigenesis. Possibly because of this, Afirma classifies 90% of the Hürthle cell neoplasms as “suspicious,” even despite the fact that the ROM in these tumors is typically lower than that for the overall Bethesda category IV group, resulting in a low PPV in these nodules. In part, this likely reflects the fact that the training of the support vector machine that encodes the proprietary algorithm included a number of Hürthle cell carcinomas but almost no benign Hürthle cell adenomas; as a consequence, the gene expression classifier determines all Hürthle cell lesions as suspicious.

Similarly, microRNA panels may inadequately classify this group of tumors. In a study, the accuracy of the panel rose from 90% to 97% when Hürthle cell neoplasms were excluded. Thus, in our opinion, Hürthle cell neoplasms should not be routinely evaluated with the currently available molecular tests, and, if used, the test results should be interpreted with caution.

Conclusions

The rapid translation into the clinical field of new discoveries in the molecular basis of thyroid cancer has led to the development of several molecular tests that address the deficiencies of thyroid cytopathology. The initial results of such tests are promising, and the rapid expansion of our knowledge in this regard promises further advances in the near future. Additional studies that independently validate these results and define the role of these tests in routine clinical practice are needed. Until the cytological classification has been further standardized, each institution interested in using these tests should determine its own risk of malignancy for each of the indeterminate categories to select the most appropriate test and to adequately interpret the results. However, in the future, molecular markers will be instrumental in refining the diagnostic accuracy of cytology and to individualize thyroid cancer management and treatment.

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Molecular testing is entrenched in the workup and management of hematological malignancies.

Biomarkers in Hematological Malignancies: A Review of Molecular Testing in Hematopathology

Mohammad Hussaini, MD

Background: Molecular interrogation of genetic information has transformed our understanding of disease and is now routinely integrated into the workup and monitoring of hematological malignancies. In this article, a brief but comprehensive review is presented of state-of-the-art testing in hematological disease.

Methods: The primary medical literature and standard textbooks in the field were queried and reviewed to assess current practices and trends for molecular testing in hematopathology by disease.

Results: Pertinent materials were summarized under appropriate disease categories.

Conclusion: Molecular testing is well entrenched in the diagnostic and therapeutic pathways for hematological malignancies, with rapid growth and insights emerging following the integration of next-generation sequencing into the clinical workflow.

Introduction
Perhaps in no other field of oncology is the routine use of molecular markers more integrated into the diagnostic, prognostic, and therapeutic workup of disease as in the realm of hematological malignancies. Molecular diagnostics is a burgeoning field in the era of personalized medicine, with high-volume laboratories running 10,000 molecular tests or more every year, many of which are for the workup of leukemia and lymphoma. Molecular testing has wide applicability in hematopathology, guiding diagnosis (eg, TCR gene rearrangement to establish T-cell clonality), subclassification (eg, recurrent cytogenetic translocations in acute myeloid leukemia [AML]), prognosis (eg, Philadelphia chromosome–positive [Ph+] in acute lymphoblastic leukemia [ALL]), and minimal residual disease testing (eg, BCR-ABL transcripts in chronic myelogenous leukemia [CML]).

Myeloid Neoplasms
Myeloproliferative Neoplasms

Chronic Myelogenous Leukemia: The Ph chromosome in CML was discovered in 1960. t(9;22) (q34;q11) juxtaposes most of ABL1 to 5′ regions of BCR, resulting in constitutively increased kinase activity and neoplastic transformation. Although the breakpoint for ABL1 is mostly conserved, occurring in the intron preceding exon 2, the breakpoints in BCR are more variable and typically occur in either

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the major or minor breakpoint regions (M- or m-bpr). M-bpr fusions result in a p210 fusion protein, which is the form typically found in Ph+ CML. A sizeable number of patients with Ph+ B-cell acute lymphoblastic leukemia (B-ALL; 40% of adults and 10% of children) also harbor the p210 product. Conversely, m-bpr translocations result in a p190 fusion found in most Ph+ B-ALL cases but rarely in Ph+ CML. Uncommonly, BCR breakpoints fall in the microregion (µ-bcr), resulting in the p230 fusion product associated with chronic neutrophilic leukemia (CNL). Detection of t(9;22) is most commonly performed by either cytogenetics, fluorescence in situ hybridization (FISH), or reverse transcription–polymerase chain reaction (RT-PCR; amplification of the transcript product); the latter is used for minimal residual disease testing.

At diagnosis, conventional cytogenetics can detect t(9;22) in 95% of cases of CML; however, an additional 2.5% of cases with submicroscopic translocations can be recovered by applying molecular methods. If the results of both cytogenetics and FISH are negative, then an alternative diagnosis should be considered.

Assaying for t(9;22) can be used to monitor the therapeutic response of imatinib mesylate and for relapse surveillance (using quantitative polymerase chain reaction [qPCR] methods, particularly screening for positive results 6–12 months after transplantation). However, the importance of the BCR-ABL1 fusion gene in CML is in its role as a paradigm for targeted cancer therapy; patients with CML may receive imatinib as first-line therapy as established by data from the International Randomized Study of Interferon and STI571 trial. Baseline values from this trial are also used as the basis of the international reporting scale (IS). The IS allows for the standardization and comparison between laboratories with regard to BCR-ABL levels.

Response to therapy can be classified as complete hematological response, complete cytogenetic response, and molecular response based on levels of fusion transcripts by RT-qPCR. A major molecular response is defined as a 3-log reduction compared with baseline (or ≤ 0.1% IS), and a complete molecular response is defined as a 4.5-log reduction or more from baseline. A lack of response may indicate acquired resistance. In such cases, Bcr-Abl kinase domain mutations (> 100 types documented) can be found in one-half of refractory cases and are an indication to adjust therapy by integrating a second-generation tyrosine kinase inhibitor into treatment.

**Atypical Chronic Myelogenous Leukemia and Chronic Neutrophilic Leukemia:** The clonality of CNL has been well established given the prior detection of 20q-, 11q-, and JAK2 V617F mutation in this disease, but they are not disease specific. Deep sequencing has identified CSF3R mutations in CNL and atypical CML in 59% of patients, and these findings were subsequently documented in all World Health Organization (WHO)–defined CNL cases, possibly prompting a future revision of the WHO diagnostic criteria.

**Polycythemia Vera, Essential Thrombocytosis, and Primary Myelofibrosis: JAK2 codes for an intracellular tyrosine kinase and provides signaling for growth factor receptors, including the erythropoietin receptor. The JAK2 V617F mutation was discovered in 2005 and was shown to be present in 95% of polycythemia vera cases and approximately 50% to 65% of cases of essential thrombocytosis (ET) and primary myelofibrosis (PMF). In addition, the JAK2 V617F mutation can also be seen in nearly one-half of cases of refractory anemia with ring sideroblasts associated with marked thrombocytosis.

In cases of polycythemia vera in which the JAK2 V617F mutation is not detected, the remaining 5% of patients may harbor mutations in exon 12 of JAK2. Similarly, in ET and PMF cases lacking the JAK2 V617F mutation, an assessment of MPL is indicated, given that 5% or more of patients with PMF and even fewer patients with ET (1%) will show an aberration in this gene (W515K/L).

Exon 10 c-MPL mutations have also been reported in ET or PMF (5%). The JAK2 V617F mutation can be detected via targeted PCR followed by sequencing of the amplicon. Other methods include restriction digest of PCR-amplified products followed by separation by capillary electrophoresis, allele-specific PCR using probe-based gene expression analysis, real-time PCR, pyrosequencing, and melting curve analysis.

Despite the discovery of JAK2 and MPL mutations, until recently many ET and PMF cases did not have a unique genetic basis (ie, JAK2, MPL) until 2 independent groups identified CALR mutations in this patient subset. CALR mutation, which comprises insertions (ins) and deletions (del) leading to a frameshift, are found in 20% to 25% of ET and PMF cases and tend to cluster in exon 9.

Commercial testing utilizes sequencing and fragment length analysis.

Recently, the Dynamic International Prognostic Scoring System Plus listed unfavorable karyotype as a risk factor for predicting survival in primary myelofibrosis.

**Mastocytosis:** Activating point mutations in KIT are highly associated with mastocytosis and can be detected in more than 95% of cases of systemic mastocytosis using real-time qPCR, allele-specific oligonucleotide PCR, or direct sequencing. KIT mutations result in the ligand-independent activation of the c-kit tyrosine kinase. The most common mutation in systemic mastocytosis is the D816V variant seen in 68% of cases of mastocytosis; however, in certain subsets (eg, aggressive systemic mastocytosis), its incidence may exceed 80%. The presence of this variant constitutes a minor criterion for the diagnosis of systemic mastocytosis.
mastocytosis. Other KIT variants have been described (< 5%) and are more likely to be detected in the context of cutaneous mastocytosis rather than systemic mastocytosis.\(^\text{20}\) Patients with the D816V variant are resistant to imatinib.\(^\text{20,35}\)

### Myeloid and Lymphoid Neoplasms With Eosinophilia and Abnormalities of PDGFRα, PDGFRβ, or FGFRI

A unique group of myeloid and lymphoid neoplasms are defined by aberrant tyrosine kinase activity due to translocations involving PDGFRα, PDGFRβ, or FGFRI, all of which are characteristically associated with eosinophilia. A workup for abnormalities in these genes should be considered in cases of eosinophilia with end-organ damage or in which secondary reactive eosinophilia has been excluded.

The cellular ontogeny of these disorders may originate from a pluripotent (lymphoid–myeloid) stem cell. PDGFRα or FGFRI can be detected with conventional cytogenetic analysis (ie, karyotype); however, the FIP1L1-PDGFRα results in an 800-kb cryptic del(4q12) that houses CHIC2. Typically, it is detected using FISH with a probe spanning CHIC2 or break-apart assay for either of the translocation partners. The translocation can also be detected using RT-PCR.\(^\text{20}\) FIP1L1-PDGFRα disease commonly manifests as chronic eosinophilic leukemia, and FIP1L1-PDGFRα is detected in 10% to 20% of those with idiopathic hypereosinophilia.\(^\text{39}\) Patients have a response to imatinib more than 100 times greater than that seen in BCR-ABL rearrangement.\(^\text{20,35}\)

Neoplasms associated with PDGFRα commonly present as chronic myelomonocytic leukemia. ETV6 is the most common translocation partner, but more than 13 others have been described; patients will be responsive to imatinib.\(^\text{34}\)

Neoplasms associated with FGFRI can manifest as acute leukemias (myeloid or lymphoid) or as chronic eosinophilic leukemia. Translocation partners include ZNF198, CEP110, FGFR10P1, BCR, TRIM24, MYO18A, HERVK, and FGFR10P2. By contrast to PDGFRα-and PDGFRβ-associated neoplasms, these disorders are unresponsive to tyrosine kinase inhibitors.\(^\text{20,34}\)

### Myelodysplastic Syndrome

Myelodysplastic syndrome (MDS) is a clonal disorder of myeloid cells characterized by morphological dysplasia and ineffective hematopoiesis that manifests as peripheral cytopenia.\(^\text{36}\) Cytogenetic abnormalities are seen in one-half of MDS cases, and they most commonly involve del(5q)/7q) or monosomies of the same.\(^\text{20,37}\) TP53 mutations are associated with therapy-related MDS and have a poor prognosis.\(^\text{38}\) Various cytogenetic abnormalities can be considered presumptive evidence of MDS even in the absence of sufficient dysplasia (ie, -5/del[5q], -7/del[7q], +8, -Y, del[20q], isochromosome [i][17q], -13/del[13q], del[11q], del[12p], del[9q], isodicentric [idic][Xq13], and certain balanced translocations involving chromosomes 1, 2, 3, 9, 11, 16, and 21).\(^\text{20}\) Various prognostic models are available for MDS. The most widely adopted is the International Prognostic Scoring System (IPSS) and its revised version (IPSS-R), both of which integrate the percentage of blasts in the bone marrow, cytogenetic abnormalities, and number of cytopenias.\(^\text{39}\) In the latter scheme, cytogenetics are placed in 5 tiers: very good (-Y, del[11q]), good (normal, del[5q], del[12p], del[20q], and del[5q] + 1 more), intermediate (del[7q], +8, +19, i[17q], and others), poor (-7, inversion [inv][3/6q/del[3q], -7/del[7q] + 1 more, and 3 cytogenetic aberrations), and very poor (> 3 abnormalities).\(^\text{40}\) Although gene-expression profiling and single nucleotide polymorphism arrays are powerful tools, they are not routinely employed in the clinical setting. However, somatic mutation in 40 genes has been found in MDS and analysis for these genes can add prognostic value.\(^\text{41}\) By contrast to cytogenetic abnormalities, which are seen in one-half of cases, at least 1 of these “driver” mutations can be found in most cases of MDS.\(^\text{42,43}\) For example, patients with 1 or more mutations in TP53, EZH2, ETV6, RUNX1, or ASXL1 show survival patterns analogous to those in the next higher tier by subgrouping in the IPSS.\(^\text{44}\)

Various epigenetic modifiers (DNA methylation regulators, spliceosome mutations, and histone modifiers TET2, IDH1/2, DNMT3A, EZH2, ASXL1, SF3B1, U2AF1, SRSF2, and ZRSR2), transcription factor genes, and kinase signaling genes have been implicated in MDS, providing a basis for approved therapies and those in development.\(^\text{45}\) However, these aberrations have limitations because their clinical significance is not always clear given their association with poor prognostic clinical features, our lack of knowledge of their interactions with other markers often concurrently detected, intratumoral clonal heterogeneity, and the wide gamut of mutations in any given gene.\(^\text{42}\)

Next-generation sequencing (NGS) technologies, which garner the power of massively parallel sequence generation, enable laboratories to clinically sequence many genes simultaneously, which was previously untenable by traditional sequencing technologies. Commercial testing is available for activated signaling genes (KIT, JAK2, NRAS, CBL, MPL), transcription factors (RUNX1, ETV6), epigenetic genes (IDH1/2, TET2, DNMT3A, EZH2, ASXL1, SETBP1), ribonucleic acid splicing genes (SF3B1, U2AF1, ZRSF2, SRSF2), and tumor suppressors (TP53, NPM1, PHF6), among others. In cases of MDS or MDS/myeloproliferative neoplasms in which the diagnoses are unclear or dysplasia has yet to emerge, detecting a mutation in one of these key genes may be helpful in establishing the diagnosis of a clonal myeloid neoplasm. Although
they are not formally incorporated into prognostic stratification schemas, certain mutations may also carry prognostic implications. In addition, robust myeloid testing commercially available (FoundationOne Heme, Cambridge, Massachusetts) can interrogate 405 cancer-related genes, allowing — in theory — for the identification of targetable mutations and patient enrollment in clinical trials.

**Acute Myeloid Leukemia**

AML is the most common type of acute leukemia occurring in adults. In 2015, an estimated 20,830 new cases of AML will occur in the United States, along with 10,460 deaths. AML is a lethal disease and has a 5-year relative survival rate of 24.2%. However, outcomes are heterogenous and overall survival rates range from approximately 5% to 70%. Thus, a need exists for prognostic markers to predict outcomes and guide therapeutic decision-making. Prognostic markers can be clinical, disease related, and molecular, although the strongest prognostic factor for predicting therapeutic response and survival is cytogenetic subgrouping.

The results of numerous clinical trials across several decades have indicated that overall survival rates can be as long as 11.5 years in favorable patient groups or shorter than 1 year in patients with adverse risk. Those with favorable risk (5-year survival rate of 50%–80%) include those with t(15;17), t(8;21), inv(16), t(1;22)(p13;q13), t(6;9)(p23;q34), TP53 deletions, and a complex karyotype, may undergo transplantation after standard induction.

Determining whether consolidation therapy is appropriate in those with intermediate risk (overall survival rate of 20%–40%) is not as clear. In this cohort, molecular testing for FLT3, NPM1, and CEBPA is informative and has therapeutic implications. For example, detecting FLT3 internal tandem duplication by PCR in patients with normal karyotype AML may lead to consolidation therapy with hematopoietic stem cell transplantation, after which patients may have a 30% likelihood of cure. FLT3 codes for a transmembrane signal-transducing protein of the tyrosine receptor kinase family and reveals 2 major abnormalities in AML, ie, internal tandem duplication in the juxtamembrane portion resulting in constitutive activation and a point mutation in Asp835 (the activity loop portion of protein) resulting in dysregulation. FLT3 aberrations are seen in 5% to 10% of AMLs. NPM1 mutations involve 4 to 11 break-point insertions in exon 12 that lead to the mislocalization of normal nucleophosmin to the cytoplasm via dimerization. This can be detected by PCR followed by sizing via capillary electrophoresis. NPM1-mutated AML has been designated as a provisional entity in the 2008 WHO classification and is associated with unique morphological (blasts with “cup-like” nuclei) features and a favorable prognosis in normal karyotype AML.

CCAAT/enhancer-binding protein α is a 42 kDa transcription factor whose loss is associated with the oncogenic transformation of myeloid cells due to a loss of differentiation. Patients with mutated CEBPA show outcomes similar to those in the favorable cytogenetic subgroup of AML (eg, t(8;21) AML). The prognostic value of CEBPA is in the double-mutated subset of patients lacking FLT3 and NPM1 mutations.

Other single gene alterations that may carry important prognostic implications have been identified in AML — many were identified during whole genome sequencing studies — and include DNMT3A, IDH1/2, TET2, WT1, ERG expression, BAALC expression, and MN1 expression, among others. IDH2 is associated with a good prognosis and TET2, ASXL1, and PHF6 confer poor prognoses. However, in the absence of prospective trials and the present controversy regarding them, many of these single genes have not been formally integrated into accepted risk-stratification models. In general, investigating the mutation status of these genes is simultaneously obtained using NGS technologies.

Biomarkers are important for subclassifying AML types, and several categories of AML are defined based on the presence or absence of recurrent genetic abnormalities alone, in particular t(8;21)(q22;q22), inv(16), t(8;21)(q22;q12), t(9;11)(p22;q23), t(6;9)(p23;q34), inv(3), and t(1;22)(p13;q13). In some cases, the detection of 1 of these aberrations alone is enough to diagnose AML, even in the absence of the conventional criteria of 20% blasts in the marrow or peripheral blood, such as in the case of t(15;17) and core-binding factor-related leukemias (eg, t(8;21)[q22;q22], inv(16)) and possibly inv(3)[t(3;3)]. These translocations can be detected by conventional cytogenetics, FISH, and more novel technologies, including single molecule imaging and NGS. One such type of sequencing uses color-coded barcodes directly hybridized to individual target molecules and then digitally detects them in a multiplexed manner.

A comprehensively targeted clinical panel currently on the market uses NGS to interrogate the exons of 405 genes and examines the intronic regions of 31 genes involved in rearrangements as well as complementary DNA (ribonucleic acid) to sequence 265 genes to detect translocations.

In some cases, the detection of a translocation...
carries both diagnostic and therapeutic importance. Namely, t(15;17)(q22;q21), which is diagnostic for acute promyelocytic leukemia (AML M3), juxtaposes the 17(q21) retinoic acid receptor α (the receptor for vitamin A involved in cell proliferation and differentiation) to the 15(q22) promyelocytic leukemia zinc finger protein (involved in transcriptional regulation and apoptosis). The chimeric protein blocks differentiation beyond the promyelocytic stage, resulting in acute leukemia. However, treatment with all trans retinoic acid allows for differentiation and, in combination with cytotoxic chemotherapy, can result in complete remission. Variant translocations involving RARA are seen in fewer than 2% of cases, but they are important given that some patients may not respond to all trans retinoic acid therapy.20,70

Another example of molecular testing informing therapeutic management involves KIT testing in AML. C-kit mutations are associated with core-binding factor AMLs and may abrogate the favorable prognosis generally associated with this group.71

**Lymphoid Neoplasms**

**Lymphoblastic Leukemia/Lymphoma**

Similar to AML, the detection of certain characteristic translocations further subclassifies cases of B-ALL, including t(12;21)(p12;q22) TEL/AML-1, t(1;19)(q23;p13) PBX/E2A, t(9;22)(q34;q11) ABL/BCR, (5;14)(q31;q32) IL3-IGH, and (Y;11)(V;q23) V/MLL.20

t(9;22)(q34;q11) is seen in 25% of adult cases and 2% to 4% pediatric cases.72 Patients with this Ph+ translocation carry the worst prognosis of all types of lymphoblastic leukemia73; however, these patients can be treated with adjuvant imatinib, which improves complete remission rates.74

The presence of the translocation also serves as a useful marker for minimum residual disease testing. Molecular methods for detecting the translocation are similar to those used for CML. Notably, when qRT-PCR testing is undertaken, the p190 protein product is typically associated with B-ALL, not the p210 protein typical of CML; in children with ALL, the break point occurs in m-bcr, which generates the p190 protein product in 90% of cases.6 If a p210 protein product is detected, then consideration should be given to a lymphoid blast crisis arising in CML.

By contrast to t(9;22), the reverse demographic appears to be true for t(12;21)(p12;q22): It occurs in 25% of pediatric cases but is rare in adults and associated with a favorable prognosis and curative rates of higher than 90% in children.75 MLL can have various translocation partners, the most common of which is AF4 on chromosome 4. MLL translocations carry a poor prognosis, and this is particularly true in infants.76 The unique characteristic of t(5;14)(q31;q32) IL3/IGH B-ALL, which is rare, is its association with eosinophilia due to the overexpression of IL3.77

t(1;19)+ B-ALL is historically associated with a poor prognosis, but this has changed through the use of intensive chemotherapy regimens.78 Immunophenotypically, the blasts lack CD34 but have aberrant CD9 positivity.79 Based on gene-expression profiling, BCR-ABL1–like B-ALL has been identified as being associated with deletions of IKZF1, CRLF2 rearrangements, and poor outcomes.80 JAK1/2-activating mutations are present in a subset of these patients and may benefit from Janus kinase inhibitor therapy.80

In all children with ALL, cytogenetic testing or flow cytometric analysis of ploidy should be undertaken. Hyperdiploidy (> 50 chromosomes) is associated with a better prognosis, whereas hypodiploidy (< 44 chromosomes) is associated with a poor prognosis.80,81

Translocations in T-cell ALL (T-ALL) commonly involve 1 of the TCR loci (A, B, G, D). The most common translocation partner includes HOX11 on chromosome 10 (occurring in 10%–30% of cases) or various other transcription factors dysregulated by juxtaposition to 1 of the TCR genes.82 PICALM-MLLT10 and MLL rearrangements are seen in approximately 10% of cases.83

Both B-ALL and mature B-cell non-Hodgkin lymphomas (NHLs) show clonal immunoglobulin (Ig) gene rearrangements, which are helpful in residual disease testing as well as in establishing a malignant diagnosis. For follow-up specimens, screening for clonal peaks identical to those identified at diagnosis can be performed to assess for residual/relapsed minimal residual disease.

B-cell antigen receptors are encoded by IGH (14q32), IGLK (2p11), and IGLL (20q11), coding for the Ig heavy chain, κ light chain, and the λ light chain, respectively. Each contains variable (V), joining (J), and constant regions; IGH contains an additional diversity (D) region. Multiplex PCR that uses primers to target highly conserved framework regions within the V segment are used to generate PCR products, which can then be separated using capillary electrophoresis, which is preferred to Southern blot analysis.84,85

Monoclonal peaks have heights 2 to 3 times that of the background and can be seen in clonal B-cell neoplasms. Repeat peaks in duplicate wells raise confidence that clonal peaks do not represent a PCR artifact. Care must be taken because false-positive results can occur in cases of benign lymphoid hyperplasia, which may be present in the setting of immunodeficiency and autoimmune disease.86,87 Furthermore, lineage infidelity is present with BCR gene rearrangement and is similar to that seen in T-cell lymphoma. Most precursor and 5% to 10% of mature B-cell neoplasms will harbor clonal T-cell gene rearrangements.6 Other various factors may result in false-negative results, including primer failure due to
somatic hypermutation (which can occur at a rate of > 50% in certain lymphoid neoplasms [e.g., follicular lymphoma]), complex IGH rearrangements, or DNA of poor quality.\textsuperscript{88}

**Mature B-Cell Neoplasms**

Various translocations are associated with B-cell NHLs and their detection helps to establish a diagnosis in these entities. t(14;18) involves BCL2 on chromosome 18 and IGH on chromosome 14. BCL2 is juxtaposed to the J region of the heavy chain. Given that the IGH enhancer element is highly active, bcl2 can become overexpressed. Because bcl2 has antiapoptotic properties, its overexpression will result in neoplasia. This translocation is found in 85% to 90% of cases of follicular lymphoma (a lower percentage occurs in cases of high-grade follicular lymphoma) and 25% of cases of diffuse large B-cell lymphoma (DLBCL).\textsuperscript{20}

Because follicular lymphomas may lack demonstrable Ig clonality due to ongoing somatic hypermutation, the use of FISH or PCR for the translocation offers alternative markers to assess for clonality, establish a diagnosis, or both; however, FISH is preferred to PCR as it is more sensitive and specific.\textsuperscript{89}

DLBCL, in addition to BCL2, can have translocations of BCL6 and MYC (10% of cases).\textsuperscript{90} When a MYC translocation is detected along with other specific translocations (usually BCL2 and BCL6) in an intermediate to large B-cell lymphoma, its presence qualifies as a “double hit” lymphoma, which may be categorized under the rubric of large B-cell lymphoma with features intermediate between DLBCL and Burkitt lymphoma.\textsuperscript{91}

Typically, MYC gene rearrangements are associated with Burkitt lymphoma, but they can also be present in plasmablastic lymphomas (50% of the time) and, rarely, in follicular lymphoma and primary central nervous system DBLCL.\textsuperscript{92,93} Burkitt lymphoma is characterized by t(8;14) involving MYC and IGH and will less commonly show translocations involving light chain loci (κ or λ).\textsuperscript{94-96} BCL6 translocations can be seen in follicular lymphoma, DLBCL, and are frequently identified in primary cutaneous leg-type DBLCL.\textsuperscript{97}

Nearly all cases of mantle cell lymphoma carry t(11;14)(q13;q32) CCND1-IGH, which can be assessed by FISH, and is preferred over PCR-based methodologies that demonstrate lower sensitivity rates (50%–60%); this is because of the large number of dispersed break points at 11q13.\textsuperscript{98}

Translocation of CCND1 with light chain has also been reported; rarely, cyclin D2 may be translocated, which should be a consideration in cyclin D1- tumors otherwise characteristic of mantle cell lymphoma.\textsuperscript{99} Various translocations have also been described in lymphoma involving the mucosa-associated lymphoid tissue. Of these, MALT1 and BCL10 translocations are worthy of mention (t[14;18][q32;q21], t[11;18] [q21;q21], t[1;14][p22;q32]) because they represent mucosa-associated lymphoid tissue that usually does not respond to Helicobacter pylori eradication.\textsuperscript{100-102}

Although multitudinous, single nucleotide variants and copy number changes have been found in B-cell NHL, sometimes even with reported prognostic significance (eg, NOTCH1 mutations in chronic lymphocytic leukemia [CLL]), in clinical practice testing for these in B-cell NHL has a limited role.\textsuperscript{103} A limited 7-gene CLL panel with targets that carry prognostic implications has been launched by Cancer Genetics (Rutherford, New Jersey).

Commonly, when molecular testing is indicated in B-NHL, the genetic aberrations are usually of diagnostic importance. BRAF V600E mutation was originally found in 100% patients with hairy cell leukemia compared with none of the 195 other peripheral B-cell lymphoma/leukemias.\textsuperscript{104-106} The results of subsequent studies have confirmed that the mutation is present in all cases of hairy cell leukemia and is rare in other chronic lymphoproliferative disorders.\textsuperscript{104-106} In lymphoplasmacytic lymphoma, MYD88 mutation has been detected with high frequency (> 90%),\textsuperscript{107} and detecting the mutation may be diagnostically useful given the overlap with lymphoplasmacytic lymphoma and other low-grade B-cell lymphomas that may be associated with plasmacytic differentiation, including marginal zone lymphoma, multiple myeloma, and CLL. In these other conditions, the prevalence of the mutation is 3% to 9%.\textsuperscript{108} Of note, nearly one-third of activated B-cell-like DLBCL harbors the mutation, and its presence is not useful in the differential with IgM monoclonal gammopathy of undetermined significance.\textsuperscript{108} Thus, a correlation with morphology and other ancillary studies is needed.

In CLL, hypermutation status is assessed by comparing each IGH clonally rearranged gene sequence with a database of germline V-region sequences to determine the expressed V-region gene and the extent and position of somatic mutations. If a difference exists of more than 2%, then the tumor is considered hypermutated and confers a better prognosis.\textsuperscript{109}

**Mature T-Cell Lymphoproliferative Disorders**

A total of 95% of T cells express the α-β receptor and a smaller proportion express the γ-δ receptor; both of these receptors contain heterodimer proteins encoded by TCR genes located on chromosomes 7 and 14.\textsuperscript{6,110} Early in development, the TCR genes undergo somatic rearrangement involving V, D, and J regions (TCRB and TCRD) or V–J rearrangements alone (TCRA and TCRG).

Unlike in B cells, in which Ig light chains (κ and λ) can be assessed for clonality by flow cytometry or immunohistochemistry, establishing the clonal na-
ture of T cells using these techniques is difficult, thus making TCR gene rearrangement studies valuable. Each T cell bears a unique, rearranged sequence. Under normal circumstances, a range of gene products can be seen given the gamut of polyclonal T cells present. However, if a clonal process is present, then a particular gene rearrangement product should predominate, and it can be detected using Southern blot analysis as a single clonal band. Although Southern blot analysis is considered the gold standard, it is inefficient and seldom used in modern clinical laboratories for T-cell clonality detection. Drawbacks of Southern blot analysis include its high cost, increased time, large sample requirements, and low sensitivity rates compared with PCR (5%–10% vs 1%).

PCR amplification of TCRG and TCRB gene products followed by gel separation or capillary electrophoresis is employed in the clinical laboratory. PCR testing demonstrates a clonal peak 2 to 3 times larger than the background peaks in T-cell lymphomas. In certain cases, false-negative results may occur if the rearrangement involves the primer site or too few T cells are present for analysis. Positive cases of gene arrangements should not be taken to mean that T-cell lymphoma is present. Such positivity can be seen in cases of B-cell lymphoblastic leukemia (approximately 50% of cases), mature B-cell lymphomas (5%–10%), AML (10%), and non-neoplastic conditions such as autoimmune disorders, certain infectious diseases (Epstein–Barr virus–induced oligoclonal processes), and certain cutaneous lesions (eg, lymphomatoid papulosis).

In ALK-positive anaplastic large cell lymphoma, t(2;5)(p23;q35) juxtaposing ALK and NPM, respectively, is the most frequent genetic translocation (83% of pediatric and 31% of adult cases) present; however, various, less frequently seen partners have also been described, including TPM3 (13%), ATIC, TGS, CLTC, MSN, TPM4, MYH9, and ALO17 (all < 1%). The translocation can be assayed using RT-PCR or break-apart FISH probes.

In T-cell prolymphocytic leukemia, the most common genetic aberration (80%) involves inv(14) juxtaposing the TRA locus at 14q11 to the TCL1A and TCL1B oncogenes. In a subset of cases, a reciprocal tandem t(14;14) is present; t(X;14)(q28;q11) has also been described but is less common. Both can be assayed using FISH. Cytogenetics can be used to detect chromosome 8 abnormalities (70%–80%), ATM deletions, as well as del(12p13), all of which can be seen in the setting of T-cell prolymphocytic leukemia.

Hepatosplenic T-cell lymphoma is associated with numerical abnormalities of chromosome 7, and most cases will demonstrate i(7q). As the disease progresses, 2 to 5 copies of i(7)(q10) or derangements in the second chromosome 7 may be present. i(7)(q10) can be detected using FISH.

In adult T-cell leukemia, clonal integration of the human T-lymphotropic virus type 1 viral DNA can be seen. Although it is conceivable to perform testing via Sanger sequencing, it is typically easier to perform serum studies for human T-lymphotropic virus type 1. In enteropathy-associated T-cell lymphoma, amplification of 19q31.3, del(16q12.1), or both have been reported.

Conclusion

Molecular testing is well entrenched in the workup and management of hematological malignancies. As sequencing technologies become both more powerful and affordable, they will take on an even larger role in the molecular diagnostics of hematopathology and in the era of precision medicine.

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The ability to isolate and molecularly analyze circulating tumor cells is becoming a reality.

Circulating Tumor Cells: A Window Into Tumor Development and Therapeutic Effectiveness
Gisela Cáceres, PhD, John A. Puskas, PhD, and Anthony M. Magliocco, MD

**Background:** Circulating tumor cells (CTCs) are an important diagnostic tool for understanding the metastatic process and the development of cancer.

**Methods:** This review covers the background, relevance, and potential limitations of CTCs as a measurement of cancer progression and how information derived from CTCs may affect treatment efficacy. It also highlights the difficulties of characterizing these rare cells due to the limited cell surface molecules unique to CTCs and each particular type of cancer.

**Results:** The analysis of cancer in real time, through the measure of the number of CTCs in a “liquid” biopsy specimen, gives us the ability to monitor the therapeutic efficacy of treatments and possibly the metastatic potential of a tumor.

**Conclusions:** Through novel and innovative techniques yielding encouraging results, including microfluidic techniques, isolating and molecularly analyzing CTCs are becoming a reality. CTCs hold promise for understanding how tumors work and potentially aiding in their demise.

**Introduction**
Cancer is a comprehensive term that includes a group of diseases characterized by host cells growing without control. It has a multifactorial origin and, even though many cancers are directly associated with risk factors in modern life, cancer is an ancient disease, with the oldest confirmation of metastatic carcinoma dating to 1200 bc.¹ Cancer is a major health problem worldwide, and 1 of every 4 deaths in the United States is attributed to cancer.² The search for biomarkers that allow early detection and may therefore affect the outcome of the disease is critical. The ability of cancer cells to spread in the body, producing metastasis, is one of the most relevant characteristics of the disease and the cause of most cancer deaths. Circulating tumor cells (CTCs) are thought to originate from primary tumors and, due to evolutionary pressures, acquire a genetic heterogeneity that gives them the potential to reach the circulation and colonize distant organs, thus gaining access to better nutrients and biological niches to enhance their survival.³,⁴ Conceptually, this idea is more than 140 years old; in 1874, De Morgan⁵ proposed that cells from a primary tumor can escape and travel through surrounding tissue along the lymphatics, or blood vessels, to invade new areas. Ashworth⁶ was the first person to observe cells in the peripheral blood that he characterized as “exactly

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likely to continue. However, because CTCs are biomarkers for malignancies, and this trend is have been extended to many types of cancer as opsy is to measure treatment effectiveness. CTCs have shown variable concordance rates. marrow compared with the corresponding peripheral blood draw is faster and less invasive than collecting a tumor sample via a surgical procedure; furthermore, some tumors are in locations that prohibit biopsies. In addition, individual CTCs may contain unique genetic information essential to understanding the tumor biology and the metastatic process of an individual patient’s tumor. This, of course, is a major oversimplification of the heterogeneous nature of tumors, CTCs, and the complexity of the metastatic process, particularly when considering that the general consensus is that CTCs are heterogeneous and can differ between different cancers as well as within an individual patient.

CTCs have been garnering attention from researchers during the previous decade, and attempts to apply discoveries about individual CTCs to the cancer clinic for individual patients is a major goal of many researchers and health care professionals. The presence of CTCs is one of many predictors of overall survival rates in patients with early stage and metastatic breast cancer, and their presence has shown to have a superior overall survival prognosis value compared with serum tumor markers, such as carcinoembryonic antigen and cancer antigen 15-3. CTCs provide equivalent or superior prognostic information as radiographic methods but without the possible health risks associated with some of them.

Disseminated tumor cells are a subtype of CTCs localized in bone marrow that may also provide prognostic information in many types of cancer. A retrospective study of metastatic breast cancer showed a significantly higher number of tumor cells in bone marrow compared with the corresponding peripheral blood stem cells, but comparative studies have showed variable concordance rates. However, bone marrow biopsy is invasive and requires special techniques; in addition, oftentimes patients experience significant discomfort during the procedure. Thus, the study of CTCs is more favorable because CTCs can be obtained through a simple peripheral blood draw.

Currently, the major advantage of liquid biopsy is to measure treatment effectiveness. CTCs have been extended to many types of cancer as biomarkers for malignancies, and this trend is likely to continue. However, because CTCs are sparse (diluted by billions of other cells) and possibly unstable in a typical blood sample, they pose technological challenges for their detection, isolation, and characterization.

Moving beyond the simple isolation and quantification of CTCs in blood samples from patients with cancer is the possibility of analyzing the functional, molecular, and genetic alterations in these cells, and applying this information to improve cancer treatment in “real time.” If realized, this possibility will revolutionize the analysis, monitoring, and treatment of cancer. Can essential information about a tumor be gathered and understood before it seeds and before the heterogeneity morphs it into another form that will lend current therapies ineffective? Many reviews cover the different aspects of CTCs, illustrating the continued interest in this field of study. The current review will briefly describe CTCs, and then focus on developments to further characterize them and the utilization of CTCs as biomarkers for cancer. We will address the potential problems of phenotypically identifying CTCs, isolating them via microfluidic techniques, and using next-generation sequencing to molecularly characterize CTCs.

**Relevance**

Currently, CTCs are used as a biomarker associated with the status of metastatic cancer. One test alone has been approved by the US Food and Drug Administration for enumerating CTCs in metastatic breast, colorectal, and prostate cancers, and that is the CellSearch System (Janssen Diagnostics, Raritan, New Jersey). The test enumerates CTCs and is commonly used before and after a given treatment. If the number of CTCs remains the same or increases, then the treatment may be ineffective; however, if the number of CTCs decreases, then the treatment may be effective. This concept is important because many studies have shown that as few as 3 to 5 CTCs in 7.5 mL of blood can lead to a poor prognosis in terms of progression-free and overall survival rates. CellSearch identifies CTCs as captured circulating cells expressing the cell-surface marker epithelial cell adhesion molecule (EpCAM; CD326), cytokeratin (CK; 8, 18, and 19), and having a positive result on the 4′6-diamidino-2-phenylindole (DAPI) nuclear stain. In addition, CTCs must be negative for the common leukocyte marker CD45, and the morphology (intact cell membrane) and size (> 4 × 4 μm²) of the CTCs must also be taken into account. Studies with the CellSearch System and others have shown that high numbers of CTCs are associated with shorter disease-free and overall survival rates. The presence of 1 or 2 CTCs in patients with nonmalignant disease or in healthy individuals has been detected, but the frequency of appearance in these conditions is rare.
Pliability

Most human solid tumors contain an heterogeneous population of cancer cells that originate from epithelial cell types; many of these are shed on a daily basis into the blood, where they survive a couple of hours. Some are apoptotic, whereas others are destroyed by the immune system, even though the immune system of patients with cancer is significantly diminished. But the most relevant patient characteristic is that few cells have the potential to form new metastases, and these cells are sometimes known as cancer stem cells. However, as documented by Plaks et al, more studies are necessary to clarify the relationship between cancer stem cells and CTCs.

Epithelial tumor cells acquire characteristics that allow them to leave the primary tumor in a process known as the epithelial–mesenchymal transition (EMT), which is normally part of embryonic development but, in cancer, is associated with an increase in invasion, metastasis, and drug resistance. The EMT process has been extensively reviewed and will be touched on here to illustrate the heterogeneity of these cells, exemplifying the challenges when identifying and isolating CTCs.

The initiation of EMT is directed by signals in the tumor cell and the surrounding non-neoplastic matrix, but the central characteristic of the EMT is associated with a decrease in members of the cadherin family, particularly E-cadherin expression, which allows tumor cells to detach and move to the surrounding areas, lymph nodes, and enter the blood vessel system as CTCs. The overexpression of N-cadherin has also been observed in prostate cancer and is associated with tumor cell motility and cancer progression in breast cancer. Moreover, evidence reveals heterogeneous cadherin switching depending on the site of origin of the carcinoma. Complex communication between different cellular events control the EMT process, including transforming growth factor β family members associated with the plasticity of epithelial cells, the overexpression of transcription factors, such as snail/slug, zinc finger E-box binding homeobox 2, forkhead box C2, goosecoid, twist family basic helix-loop-helix transcription factor 1, and activation of members of the Src tyrosine kinase family. EMT cells in breast cancer show resistance to conventional therapies and surface markers associated with stem cells CD44+/CD24−/low. The surviving CTCs travel through the circulatory system to distant organs where the reciprocal process of the mesenchymal–epithelial transition allows them to invade the tissue.

Some tissues may be predisposed to metastasis to specific cancers, which Paget stated as early as 1889: “In cases of cancer of the breast, it is strange how often the liver is the seat of secondary cancer.” Indeed, the bone, liver, lungs, and brain are all sites for metastatic dominance. Currently, Paget’s “seed and soil” hypothesis is still valid, but metastasis is now recognized as a dynamic process whose outcome depends on the interfaces with the surrounding homeostatic mechanisms that will differentiate the cell from the original tumor. Even with metastasis, which is normally assumed as a late-stage process after the tumor reaches considerable size, evidence exists that invasion could occur in the early stages of disease and then stay clinically dormant.

Novel Isolation Techniques

CTCs are difficult to isolate because so few are present in the general circulation; in addition, blood is a complex liquid tissue composed of billions of red corpuscles and hundreds of millions of white blood cells per milliliter, thus presenting technical challenges for the identification and isolation of CTCs. Many common methods for the enrichment of these rare cells have been previously reviewed, and a summary of these select techniques is shown in the Table. Because many of these techniques have been reviewed elsewhere, we will focus on novel CTC isolation techniques primarily using microfluidics that decrease CTC cell loss, increase recovery rates, use small volumes of blood, and enable the molecular analysis of individual CTCs. Their current advantages and disadvantages are also reviewed.

Two recent studies evaluated the ability to capture and genetically analyze CTCs through gravity-fed microfluidic devices. The first used photolithography and deep-reactive ion etching to create a micronetwork of cell-trapping chambers of 20 × 25 × 30 microns containing 8 to 10 micron pore channels. From this master silicon template, a soft, elastomeric negative mold was forged by pouring and curing against the silicon master. The final microsubstrate was created by hot embossing a plastic plate made of cyclic olefin polymer against the elastomeric negative mold. A thin plastic laminate was then laminated against the cyclic olefin polymer to create the final microchip that allowed blood cells to flow through, leaving the larger CTCs in the chamber. Using the breast cancer cell lines MCF7, MDA-MB-231, and SK-BR-3 spiked into phosphate-buffered solution and human blood, the researchers captured more than 80% of the CTCs. They were also able to show the differential expression of the cancer biomarkers PanCK, ERBB2 (formally known as HER2), ER, PI3K, E-cadherin, and vimentin in the cell lines tested by immunostaining antibodies and fluorescence microscopy.

The second study was conducted by a group from Japan who developed a novel, rapid CTC isolation device using a 3-dimensional palladium filter. The filter was produced by microfabrication technology and consisted of lithography and electroforming pro-
cesses forming 8 micron pores that allowed normal blood cells and leukocytes to flow through while larger CTCs remained in the pocket. Cell-spiking experiments showed a 90% recovery rate across a range of 50 to 5,000 green fluorescent protein-tagged COLM-5 tumor cells. The filter detected and enumerated CTCs from 19 individuals with metastatic breast cancer, 12 individuals with nonmetastatic breast cancer, and 12 healthy volunteers. The average numbers of CTCs detected were 3.37, 0.23, and 0, respectively, suggesting that the filter may have clinical utility.

Using COLM-5 cells, the researchers also showed that further analysis could be performed on the filter using immunohistochemistry for ERBB2, ER, and PGR, and genetic analysis for EGFR/KRAS mutations by polymerase chain reaction (PCR), whole genome amplification, and direct sequencing.

The major advantages of these techniques are the capture of EpCAM-negative CTCs and the capability of enabling single CTC genomic analysis. Two major dis-
advantages of these methods are that the US Food and Drug Administration has not yet approved them for use and they lack the validation required to be used in a clinical setting. In addition, size filtration-based approaches similar to the 2 reviewed here can have significant red and white blood cell contamination if the pores are plugged with CTCs. This could hinder downstream genetic analysis by increasing the DNA background contamination.

Because EpCAM expression may be lost during EMT, microfluidic devices are being developed to capture CTCs — in particular, EpCAM-negative CTCs — by other means. Galletti et al developed a geometrically enhanced, differential immunocapture microfluidic device using anti-ERBB2 antibody-coated microposts to isolate CTCs (DAPI+, CK+, CD45–) from patients with breast and gastric cancers. Notably, ERBB2 expression is correlated with poor prognosis and plays an important role in the metastasis and invasion of breast cancer to surrounding tissues during EMT. The researchers optimized their system using breast cancer cell lines and tested it in individuals both positive and negative for ERBB2 and found CTCs. This may be an alternative method for evaluating EpCAM-negative CTCs. The same system has also been developed for prostate cancer using a monoclonal, anti–prostate-specific membrane antigen antibody.

A novel, high throughput spiral microfluidic device with a trapezoidal cross-section that relies on the microchannel dimensions and fluidic forces for the size-based separation of CTCs was developed and tested in patients with metastatic breast and lung cancers. The device has a low cost and can process 7.5 mL of peripheral blood in 8 minutes, and its capture efficiency rate is approximately 80% using spiked MCF7, T24, and MDA-MB-231 cell lines. Analysis of patient samples revealed CD24+CD44+ and CD24−CD44− cancer stem cell subpopulations and EpCAM+pan-CK+ cells, which are not currently identified in the traditional EpCAM-positive CTC capture methods. The system is also amenable to fluorescence in situ hybridization and other molecular techniques following CTC isolation.

Sheng et al developed a geometrically enhanced, mixing, high-performance microchip for the efficient capture of pure CTCs. After testing with anti-EpCAM antibody–coated surfaces within the specialized staggered herringbone grooves in the microchip, they found an optimal flow rate of 1 μL/second, a flow velocity of 0.75 mm/second, and a maximum sheer stress of 0.38 dyn/cm. The researchers achieved a capture efficiency rate of 90% of the EpCAM-positive human pancreatic cancer cell line L3.6pl. After spiking between 50 and 50,000 labeled L3.6pl cells into lysed or whole blood, they achieved a 92% capture rate from lysed and a 89% capture rate from whole blood. This method of CTC visual microscopie enumeration was also amenable to isolation via trypsinization and high flow rate washing, recovering more than 60% of the CTCs with a high viability for either CTC culture or further characterization. However, a drawback to this method was that it showed high levels of contaminating leukocytes, especially from patients with pancreatic cancer (> 24,000/mL) compared with healthy donors (~ 3,500/mL).

An improvement of existing technology uses microscale vortices (Vortex Chip [University of California, Los Angeles]) and inertial focusing (which uses fluid inertia through shaped microchannels to align cells at high flow rates) for the high-purity isolation of the relatively larger CTCs from whole blood. By changing the flow rates, channel heights, and length of the Vortex Chip, researchers optimized the system with 19 micron diameter particles. Using 7.5 mL of blood from 8 individuals with breast and lung cancers, they isolated CTCs (23–317 per 7.5 mL) and reduced leukocyte contamination (57%–94% purity). The major advantages of this improved technology over current technologies are reduced processing time (20 minutes), the applicability of the system to other types of cancer, and maintaining the integrity of CTCs for downstream molecular analysis.

Which one of these isolation methods, if any, will be appropriate for health care professionals evaluating patients with cancer remains an open question. What is clear is that the isolation of these rare cells is imperative for the molecular analysis of CTCs and, ultimately, the personalized patient care for all cancer types.

Limitations
The main limitation of the CellSearch System is that the isolation method utilizes EpCAM expression on the cell surface of the tumor, which is expressed in 75% of cancer types alone (and may be lost in undifferentiated tumors or subclones). In renal cell carcinoma subgroups, EpCAM is expressed in chromophobe, papillary, and oncocytoma types but is significantly absent in clear cell carcinomas, whereas others report decreased EpCAM expression in metastases. Similarly, anaplastic thyroid carcinomas lack EpCAM expression, whereas differentiated and poorly differentiated thyroid carcinomas show overexpression. Brain metastasis from breast cancer has been found without the presence of EpCAM-positive CTCs. Similarly, in different human carcinomas, an approximately 10-fold lower rate of EpCAM expression has been found in CTCs than in primary and metastatic tissues. A significant association exists between positive EpCAM expression and survival rate in esophageal cancer, and clear-cell renal cell carcinoma, moderately differentiated colon cancer, and thyroid carcinoma; however, EpCAM expres-
sion has also been associated with a worse prognosis in different types of breast cancer, pancreatic cancer, gallbladder carcinoma, esophageal squamous cell carcinoma, and urothelial carcinoma of the bladder. For epithelial ovarian cancers, lung cancer, prostate cancer, and well and poorly differentiated colon cancers, no clear correlation with survival rates has been observed. These contradictory data highlight the need to discover other phenotypic markers to more precisely identify CTCs. The CellSearch method could use alternative capture approaches beyond antigens to EpCAM, but these methods would be considered experimental approaches and not readily available to patients.

**Molecular Characterization**

Many methods are used to molecularly characterize CTCs, including those that are protein- or nucleic acid-based (eg, reverse transcription–PCR), as well as microarray analysis and sequencing. These methods and the variety of iterations within each general method have been reviewed in detail by Lowes et al. Thus, this review focuses instead on massively parallel or next-generation sequencing and the practical applications pertaining to patient care and personalized medicine.

The method of molecular characterization is generating interest because of the multiplexing capability and the generation of clonal evolution information that may point to a key player in the progression of cancer. After enumeration and classification, delving into the genetic makeup of CTCs may be important because such data may provide additional information on the mutational status of the tumor. Although the technologies used to isolate individual CTCs are technically demanding, they are beginning to take shape and become available. The technology now exists to look into the genome of individual CTCs, potentially discovering the genetic drivers that allow tumors to progress and evade therapy. Learning more about the genetic makeup of a tumor may allow researchers to discover new and potentially important driver mutations, give biotechnology investigators the information they need to develop novel drugs that target those mutations, and inform health care professionals on how to better treat and cure more patients with cancer.

However, this is a formable task; simply knowing that a DNA mutation exists does not necessarily mean it is important for tumor development or that it is the only mutation within the tumor. Temporal, spatial, and biological considerations, among many others, may affect the way an individual tumor is developing, but even this view may be too narrow. There are caveats. For example, given that primary and metastatic tumors as well as CTCs are generally heterogeneous, how many individual CTCs should be characterized to achieve a reasonably confident estimate of the make-up of CTCs in the circulation? Which genetic profile — if there are multiple variations as there undoubtedly will be — represents the most meaningful mutational “snapshot” of the tumor? Which driver mutations will give individual CTCs the selective advantage to seed and create new metastases? These and many other questions must first be answered.

In a molecular analysis of comprehensive genomic profiling of CTCs from patients with stage 4 colorectal carcinoma using array comparative genomic hybridization (CGH) and next-generation sequencing, researchers discovered that the primary tumor differs from a liver metastatic tumor, which also differs from CTCs. When deep sequencing the primary tumor, they determined that mutations thought to be unique to CTCs instead originated from small subpopulations within the primary tumor.

As proof of concept, Lohr et al established a standardized process for isolating, qualifying, and retrieving pure CTCs in a study of metastatic prostate cancer; they also generated and quantified independent libraries for whole exome sequencing from magnetically isolated CTCs. This approach looked to find mutations in early tumor evolution (early trunk) or metastatic precursor (metastatic trunk) mutations but did not emphasize private, somatic, single-nucleotide variant mutations from single CTCs. Rather, the researchers used consensus-based variant calling to obtain a combined 19-CTC composite of the CTC population. The process worked well for individuals with cancer who had more than 5 CTCs per 3.75 mL of blood and generated high-quality libraries for whole exome sequencing. The researchers were also able to identify 10 early trunk mutations, including TP53,
found in the primary tumor and metastasis; 90% of these were found in the CTCs analyzed. This finding implies that the cancer came from a single ancestor and then diverged. Lohr et al also found 56 metastatic trunk mutations present in the primary tumor and metastasis, and 73% of these were also found in the CTCs.

Magbanua et al developed a new CTC isolation method based on immunomagnetic enrichment via the CellSearch System and fluorescent-activated cell sorting followed by genomic analysis, including profiling copy number variation (CNV) by CGH and whole genome amplification. CTCs from 9 individuals with castrate-resistant prostate cancer were successfully profiled and had multiple copy number aberrations, including gains in 8q and losses in 8p, as previously reported. The androgen receptor locus had high CNV in 78% of the cases. Similarly, in metastatic breast cancers, CTCs in 102 of 181 individuals were profiled and revealed a wide range in CNV; serial testing of the CTCs was also performed that confirmed reproducibility and indicated genomic changes over time. In addition, an array CGH analysis confirmed that primary tumors and nodal metastasis were highly correlated with each other but less so with CTCs, suggesting that a CTC copy number analysis might provide information about the progression of the tumor in real time. This can be advantageous for health care professionals who may need to change therapeutic interventions based on the genetic signature of the CTCs.

In a study of individuals with colorectal cancer, a group of researchers isolated single CTCs using a micromanipulator and showed by array CGH, mutational profiling, and microsatellite instability analysis that heterogeneity exists between tissue and CTCs and single CTCs. In addition, transcriptional analysis revealed a down regulation of epithelial markers (EpCAM, CK19, and carcinoembryonic antigen), CTC dormancy (reduced Ki67 and c-Myc expression), and an immune escape phenotype (overexpression of CD47, an antiphagocytic signal expressed on cancer cells that signals to macrophages and dendritic cells) compared with the corresponding tumor tissue. This analysis highlighted the survival mechanisms of CTCs and their ability to escape the immune response against tumors, a signature event in the metastatic process.

In patients with lung cancer, reproducible CNV patterns can be found from single CTCs using multiple annealing and looping-based amplification cycles. Study findings suggest that gains and losses of copy numbers at certain chromosome regions during metastasis are selected for and supply the tumor cells with a survival advantage in the circulatory system. The study researchers also found that a small number of individuals with lung adenocarcinoma (N = 4) exhibited nearly identical global CNV patterns (78% of the gain and loss regions were shared). This finding could suggest the possibility that CTC CNV analysis may be a biomarker for lung adenocarcinoma.

It is likely that the genetic analysis of CTCs will be an important area in the development of biomarkers for cancer. However, questions still remain about whether circulating tumor DNA (ctDNA) may be a better or more useful biomarker than CTC analysis. Evidence is accumulating, and analysis of 62 normal and clinically matched samples showed that CTC DNA returned actionable mutations 3 times more often than ctDNA, and ctDNA produced 7 times more false-positive results and 50 times more variation in sensitivity rates than CTC DNA. These are preliminary data; more studies are needed before such a question can be fully answered.

However, it is likely that ctDNA will be a complementary assay to CTCs, providing information on the global tumor load in a patient and the indicators of the genomic conditions of active and dormant cancer cells undergoing apoptosis. In this setting, CTCs more likely represent a real-time glimpse of the biological and functional condition of viable malignant cells and may provide opportunities for a pharmacodynamic evaluation and rapid treatment switching to optimize therapy for patients with cancer.

Conclusions

Circulating tumor cells (CTCs) play a key role in the metastatic process. Ever since the CellSearch System was first approved by the US Food and Drug Administration for the enumeration of these rare cells and was shown to have prognostic value, emphasis has been placed on using these cells to further evaluate tumors. The ability to have a noninvasive, therapeutically relevant diagnostic test has great appeal for health care professionals and patients alike. Discovering the genetic potential of these cells will no doubt lead to a better understanding of the evolution of tumors in patients with cancer.

The future of CTC research is bright. As researchers develop more precise CTC phenotypic markers, such as ERBB2, EGFR, heparanase, and notch1, a signature found in CTCs of metastatic breast tumors that colonize the brain, the ability to further delineate CTC subsets will become a reality. Although the concept of CTCs is not a novel one, this field of study is still in its infancy. With the development of better isolation methods, coupled with improvements in technologies that allow for the genetic characterization of CTCs, new discoveries about how tumors progress, their metastatic potential, and the time-dependent nature of tumor progression will most certainly occur. However, to achieve reliable and comparable results, researchers must standardize the types of isolation methods they use. It is likely that different isolations methods, with unique limitations, could
give different results.

Many more questions still need answers to realize the potential of CTCs. Because CTCs are a heterogeneous population of cells, single-cell genetic analysis will be required, as will searching the profile(s), determining the metastatic potential of the tumor cell, and understanding the copy number variations of specific genes known to be critical in CTCs. In their study of lung cancer, Ni et al illustrated that all CTCs from a given individual, irrespective of cancer subtype, showed reproducible copy number variation patterns similar to the metastatic tumor in the same individual.

An intriguing advantage of the liquid biopsy and CTC analysis is the ability to serially sample and monitor in real time. Until now, health care professionals have seen snapshots of the tumor, instead of the full picture, and the “average” profile. Monitoring the progression of cancer is a significant advantage for health care professionals who may change therapeutic interventions in their patients based on real-time information. If a treatment course has failed (based on the elevated CTC level), then the patient could be switched to another therapy earlier.

In addition, detailing the genetic makeup of CTCs over time may allow personalized medicine to become a reality by addressing specific mutations rather than using chemotherapeutic approaches that do not selectively kill all tumor cells. It is unlikely that an analysis of CTCs will be the answer to all of the biological questions related to tumors, but it is possible that analyzing CTCs may unlock enough information about them to arm health care professionals with data that could improve quality, efficacy, and effectiveness of therapies aimed at eradicating cancer.

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Surgical and Molecular Pathology of Barrett Esophagus
Sherma Zibadi, MD, PhD, and Domenico Coppola, MD

Background: Patients with Barrett esophagus (BE) are predisposed to developing dysplasia and cancer. Adenocarcinoma, which is associated with BE, is the most common type of esophageal tumor and, typically, it has an aggressive clinical course and a high rate of mortality. Methods: The English-language literature relating to tumor epidemiology, etiology, and the pathogenesis of BE was reviewed and summarized. Results: The role of pathologists in the diagnosis and pitfalls associated with grading Barrett dysplasia is addressed. Current molecular testing for Barrett neoplasia, as well as testing methods currently in development, is discussed, focusing on relevant tests for diagnosing tumor types, determining prognosis, and assessing therapeutic response. Conclusions: Grading is essential for developing appropriate treatment plans, follow-up visits, and therapeutic interventions for each patient. Familiarity with current molecular testing methods will help physicians correctly diagnose the disease and select the most appropriate therapy for each of their patients.

Introduction
Barrett mucosa refers to a metaplastic process induced by the acid-peptic content of the stomach that then erodes the esophageal squamous mucosa. During endoscopy, changes of any length recognized as columnar-type mucosa in the distal esophageal epithelium that, on biopsy, are confirmed to have intestinal metaplasia are also defined as Barrett mucosa. Barrett esophagus (BE) is more common in men and has a male:female ratio of 3:1 to 7:1. Although BE can present at any age, its peak incidence occurs in the sixth decade of life and it occurs more frequently in whites (80% of patients) than patients of other races. Patients with BE are predisposed to esophageal adenocarcinoma (EAC; 5-year survival rate of 14%-22%), which has increased in incidence during the last 30 years. Guidelines for diagnosis and surveillance intervals for patients diagnosed with BE have been established and developed by the American College of Gastroenterology (Table 1).

Etiology and Pathogenesis
Typically, patients acquire BE as a result of gastroesophageal reflux. The factors putting patients at risk for BE are several and include duodenogastric
reflux, which is a delay in the clearance of acid juice from the esophagus, a resting pressure in the sphincter of the lower esophagus less than the optimal rate, and the presence of hiatal hernia.\(^5\)\(^-\)\(^11\)

Other etiological factors include postgastrectomy bile reflux\(^12\) and esophageal injury (eg, lye ingestion, tobacco use).\(^13\)\(^,\)\(^14\) Alcohol intake does not promote BE\(^15\) and Helicobacter pylori infection has a protective effect, particularly if Helicobacter possesses the CagA gene.\(^16\) In infants, BE may arise from congenital rests of gastric columnar epithelium reacting to gastroesophageal reflux.\(^5\)\(^,\)\(^17\)

The way in which normal squamous epithelium transitions into specialized columnar epithelium is not yet clear.\(^18\) However, based on the theory of restitution and replication proposed by Jankowski et al,\(^19\) after the acid-peptic content of the stomach erodes the parabasal and superficial layers of the squamous mucosa (Fig 1A), the basal progenitor cells move into denuded areas.\(^5\) In the setting of persistent gastroesophageal reflux, these multipotent stem cells become resistant to acid and bile, selectively differentiating into columnar epithelium that secretes mucin (Fig 1B).\(^5\)\(^,\)\(^19\) By contrast, the upward movement of the columnar epithelium from the stomach could cause metaplasia due to wound healing of the ulcerated mucosa.\(^5\)

**Cancer Risk**

BE is a premalignant condition associated with EAC and dysplasia.\(^20\)\(^,\)\(^21\) During the last several decades, the incidence of EAC has risen.\(^21\)\(^-\)\(^25\) Approximately 10% to 20% of patients with symptomatic gastroesophageal reflux who undergo endoscopy have BE.\(^24\) However, many patients with BE are asymptomatic.\(^25\) The prevalence of dysplasia in the setting of BE varies between 14% and 40%,\(^26\) and 7% to 15% of patients will have EAC at the time of initial diagnosis of BE.\(^27\)

**Pathology**

Grossly, the esophagogastric junction in BE is displaced by areas of velvety, gastric-type mucosa that macroscopically appear salmon in color.\(^11\) These areas of gastric-type mucosa proximally extend from their typical location. The area of BE may be ulcerated or contain islands of residual, whitish squamous mucosa surrounded by pink-colored, gastric-type mucosa (Fig 2). It is possible that the health care professional may be unable to distinguish the appearance of simple BE — grossly and endoscopically — from BE with early cancer and dysplasia.\(^28\) With regard to its location, BE characteristically involves the lower one-third of the esophagus, but it may involve the middle and upper esophagus as it progresses.\(^11\)

Previously, BE was histologically indicated by the presence of intestinal metaplasia or the gastric, cardia, or oxyntic type of columnar mucosa in the esophagus.\(^29\)\(^,\)\(^30\) However, in the modern era of endoscopy, the American Gastroenterological Association requires the presence of unequivocal intestinal metaplasia for there to be a diagnosis of BE.\(^31\)\(^,\)\(^32\)

The nonintestinalized, gastric-type mucosa was previously thought to have no potential for malignant transformation; however, in recent years, small (presumably early) EACs arising in the absence of intestinal metaplasia have been reported.\(^33\)\(^-\)\(^35\) Thus, these findings suggest that the identification of goblet cells as a requirement for diagnosing BE should be challenged.\(^26\)\(^-\)\(^28\) For example, the requirement no longer exists in Japan, which was the first country to propose the existence of malignant potential for nonintestinalized BE.\(^35\)\(^,\)\(^38\) In recent years, this view has been supported by study findings indicating that goblet cell–containing epithelium has similar DNA abnormalities as metaplastic esophageal columnar epithelium without goblet cells.\(^39\)\(^,\)\(^40\)

Although goblet cell metaplasia is not always an obligate precursor of malignancy, it is important to point out that the recognition of goblet cell metaplasia remains the most important criterion in clinical practice for the routine histological recognition and diagnosis of BE.

In BE, the intestinalized mucosa rarely has a villiform appearance and Paneth cells, absorptive cells, and intestinal-type endocrine cells are typically absent because of the incomplete form of metaplasia present. Uncommonly, the complete form of metaplasia presents in conjunction with the usual incomplete form of metaplasia.

Shaped like barrels, goblet cells have a distended

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**Table 1. — ACG Guidelines for BE**

<table>
<thead>
<tr>
<th>Dysplasia</th>
<th>Diagnosis</th>
<th>Follow-Up</th>
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</thead>
<tbody>
<tr>
<td>None present</td>
<td>2 EGDs with biopsy in 1 y</td>
<td>EGD every 3 y</td>
</tr>
<tr>
<td>Low grade</td>
<td>Highest grade on repeat EGD with biopsies within 6 mo</td>
<td>1-y interval until no dysplasia (× 2)</td>
</tr>
<tr>
<td>High grade</td>
<td>Mucosal irregularity</td>
<td>Continued 3-mo surveillance or intervention based on results and outcomes</td>
</tr>
<tr>
<td>Invasive adenocarcinoma</td>
<td>Expert pathology confirmation</td>
<td>Esophagectomy</td>
</tr>
</tbody>
</table>

ACG = American College of Gastroenterology, BE = Barrett esophagus, EGD = esophagogastroduodenoscopy, AM J Gastroenterol, 103(3):788-797, copyright 2008.
Fig 1A–C. — (A) Basal progenitor cells move into denuded areas after the gastric acid-peptic content from the stomach has eroded the squamous mucosa. (B, C) Persistent gastroesophageal reflux causes multipotent stem cells to differentiate into columnar, mucin-secreting epithelium resistant to acid and bile. Reprinted from Am J Pathol, 154(4), Jankowski JA, Wright NA, Meltzer SJ, et al, Molecular evolution of the metaplasia-dysplasia-adenocarcinoma sequence in the esophagus, 965-973, Copyright 1999, with permission from Elsevier.
cytoplasm filled with acidic mucin and can be identified using hematoxylin and eosin stain, and, if necessary, also highlighted with either Periodic acid–Schiff or Alcian blue (pH 2.5) stain.

Role of Pathologists
The role of pathologists when dealing with esophagogastric biopsy or resection is twofold:

1. Identification of BE: The pathologist must differentiate between nonmetaplastic columnar epithelium (eg, hiatal hernia, ectopic gastric mucosa), intestinal metaplasia of the cardia, and true BE.
2. Identification and grading of dysplasia: This task will affect patient treatment.

When dealing with endoscopic mucosal resection specimens, pathologists are responsible for assessing the resection margins. If invasive cancer is present, then pathologists are also required to report the distance of the invasive carcinoma from the deep margin, the presence of angiolymphatic invasion, and, if possible, the level of invasion within the muscularis mucosa, submucosa, or both.

Dysplasia
The presence of the neoplastic epithelium confined within the basement membrane of the gland from which it arises and within the superficial layer of epithelium is the defining characteristic of dysplasia. Criteria have been established for grading dysplasia in BE that consider a combination of features, such as gland architecture, the presence of inflammation, ulceration, or erosion, cytological features, and the level of maturation of the surface epithelium.

Negative
When cases of BE are negative for dysplasia, the underlying glands will not be as mature as the surface epithelium. The nuclear:cytoplasmic ratio is lower than that of the deeper glands. Lamina propria is abundantly present between the glands. Cytologically, nuclear polarity is retained, the nuclear membrane is smooth, and the nucleoli are inconspicuous. In the event of prominent inflammation, reactive changes will be present but surface maturation is maintained.

Indefinite
A diagnosis of indefinite for dysplasia is used for cases with worrisome changes but not diagnostic for dysplasia, typically because of the presence of marked inflammation. Normal glandular architecture may be observed but crowding will be present, and, cytologically, nuclear hyperchromasia, nuclear membrane irregularities, and increased mitoses at the base of the glands (but with surface maturation) may be present. Nuclear polarity is not lost in cases of indefinite for dysplasia. Such a diagnosis may also be used in the presence of tangential embedding that does not allow for the assessment of the superficial portion of the glands.

Low Grade
In cases of BE with low-grade dysplasia (LGD), the mucosal surface is similar to the underlying glands, which reveal nuclear stratification and nuclear polarity. Glandular crowding can be seen, but the lamina propria is still present between the glands. The nuclei are elongated, hyperchromatic, and have irregular contours and sustain a moderate increase in mitotic activity. The nuclear:cytoplasmic ratio will be mildly increased. In the setting of LGD, minimal inflammation may be present and nucleoli are not prominent.

High Grade
In the setting of BE with high-grade dysplasia (HGD), surface maturation is lacking; glandular distortion and glandular crowding are both present with a minimal amount of lamina propria between the glands. The glands may become dilated with luminal necrotic debris. The nuclei display marked enlargement, anisocytosis, pleomorphism, hyperchromasia, membrane irregularity, and prominent nucleoli. The mitotic figures are numerous and the nuclear:cytoplasmic
The ratio will be large. Nuclear polarity is lost (Fig 4). Inflammation will be minimal. In the setting of HGD, the presence of superficial ulceration, cytologically high-grade nuclei, solid nests of dysplastic cells with multiple secondary lumina, and/or dilated dysplastic tubules containing granular eosinophilic and nuclear debris may indicate an unsampled invasive carcinoma. Additional biopsies should be performed when observing these findings.48,49

Clinical Significance
One study found that HGD was a marker of metachronous or synchronous adenocarcinoma in approximately 40% of cases.50 In another study, Weston et al51 found that HGD progressed to adenocarcinoma or multifocal HGD in 53% of cases.

Pitfalls Associated With Grading
The grading of dysplasia in BE is subjective due to sampling errors as well as interobserver and intraobserver variations. In addition, the biological behavior of the lesion and its degree of dysplasia may not correlate.34,52 However, the task of grading dysplasia in BE is important and can sometimes have crucial therapeutic implications (see Table 1).5 For this reason, biopsy findings in cases of BE, particularly in the setting of HGD and carcinoma, should be reviewed by a second pathologist with expertise in gastrointestinal pathology.53

Morphological and molecular data suggest that dysplasia in BE begins at the base of the crypts.54 Crypt dysplasia is thought to progress into the remaining portion of the crypt and surface epithelium.55 Histologically, crypt dysplasia may exhibit the same cytological changes characteristic of LGD or, rarely, have high-grade cytological features with very large nuclei, a large nuclear:cytoplasmic ratio, irregular nuclear membranes, nuclear polarity loss, and eosinophilic cytoplasm. One study had good interobserver agreement when diagnosing crypt dysplasia.55

Intramucosal Carcinoma
In intramucosal adenocarcinoma, the neoplastic cells are limited to the mucosa (Fig 5); however, these cells will make their way to the basement membrane and
into the muscularis mucosae or the lamina propria, but they do not go beyond this point. Histologically, an effacement of the architecture of the lamina propria can be seen, as well as back-to-back microglands, small clusters, or infiltrating single cells. A syncytial growth pattern may also be observed. Typically, desmoplasia is absent or not completely developed, perhaps accounting for the poor intraobserver reproducibility in the diagnosis of this entity.

**Invasive Adenocarcinoma**

Invasive adenocarcinoma has a variable gross appearance and size (≤ 10 cm) and typically involves the distal one-third of the esophagus. Tumor cells infiltrate beyond the muscularis mucosa in the setting of invasive adenocarcinoma and oftentimes evoke a desmoplastic reaction. The microscopic pattern is mostly of the intestinal form, however diffuse, and signet ring cell or mixed types can be observed. The tumor cells may produce mucin, and the tumor itself will generally be well to moderately differentiated.

**Intestinal Metaplasia of the Cardia**

Intestinal metaplasia of the cardia has a prevalence of 6% to 25%. Generally in this setting, the cardia contains areas of intestinal metaplasia with goblet cells and a normal Z line. *H. pylori* infection, gastroesophageal reflux, and carditis can all be causes for intestinal metaplasia of the cardia. Patients with intestinal metaplasia of the cardia are at increased risk for gastric cardia cancer; however, this type of intestinal metaplasia may go into regression if its cause is treated; furthermore, research suggests that this condition is not typically associated with dysplasia.

**Endoscopic Mucosal Resection**

Gastroesophageal endoscopic mucosal resection is a procedure to remove dysplastic or carcinomatous mucosa from the stomach, esophagus, or both. It is performed on low-risk adenocarcinomas using either an injection- or cap-assisted endoscopic mucosal resection. Low-risk adenocarcinoma is defined as a stage 1A tumor no larger than 20 mm in size without vascular or lymphatic invasion and a histological grade of G1 or G2.

Following endoscopic mucosal resection, a complete tumor response was reported in 99% of patients after a follow-up time of 36.7 months. Recurrent or metachronous lesions were present in 11% of patients and the 5-year survival rate was 98%.

**Molecular Alterations**

Jankowski et al. highlight restitution, cell cycle and apoptosis, adhesion, DNA instability, and invasion as potential factors in the molecular pathways associated with the progression of BE to adenocarcinoma. For example, following mucosal injury, the mucosa rapidly heals by restitution. During this phase, metaplastic clones with specialized intestinal metaplasia propagate in the presence of persistent gastroesophageal reflux, followed by the loss of cell-cycle checkpoints. In addition, because of increasing proliferation, genomic instability is thought to be associated with slow clonal expansion. The inhibition of apoptosis occurs late and only in cases of HGD. Altering cell adhesion can precede invasive cancer, and the accumulation of genetic errors that follows may be associated with the generation of multiple, transformed cell clones, thus expanding the number of altered cells with metastatic or angiogenic potential.

**Aberrant p53**

Several studies have observed alterations in p53 during the progression of BE. Typically, aberrant p53 is accumulated in the nucleus of the neoplastic cells, particularly in cases of HGD. When they were detected by polymerase chain reaction single-strand conformation polymorphism, the p53 molecular alterations coincided with the level of p53 protein overexpression detected by immunohistochemistry in Barrett adenocarcinoma and BE-HGD but were discordant in BE-LGD. Currently, the only accepted predictor for progression is a histological diagnosis of LGD; its predictive value is low. However, the immunohistochemistry status of p53 has diagnostic value and can help predict neoplastic progression in patients with BE. Using more than 12,000 biopsy samples from 635 patients, including those with BE who developed HGD, those with invasive adenocarcinoma, and those without neoplastic progression, Kastelein et al. studied the protein expression of p53 via immunohistochemistry. The researchers found that 49 patients (8%) developed HGD or invasive adenocarcinoma during the follow-up period. The results of the study indicated that patients with a diagnosis of LGD had a
positive predictive value for neoplastic progression of 15%. Conversely, patients with LGD and concurrent altered p53 expression had a positive predictive value of 33%. Thus, these data show that p53 alterations are a better predictor of tumor progression than BE with LGD.

ERBB2 Amplification and Overexpression

Using a tissue microarray, Hu et al looked at 34 cases of BE, 18 cases of LGD, 15 cases of HGD, and 116 cases of EAC to study ERBB2 (formerly known as HER2 or HER2/neu) amplification using chromogenic in situ hybridization. They found ERBB2 amplification in 21 of the cases of EAC (18.1%), ERBB2 overexpression in another 14 cases of EAC (12.1%), and ERBB2 protein overexpression in 1 case of HGD (6.7%). All of the cases of LGD and BE had negative results for fluorescence in situ hybridization (FISH) and immunohistochemistry.

A recent study looked at the immunohistochemical expression of ERBB2 in BE, BE with dysplasia, and adenocarcinoma. The overexpression of ERBB2 in a significant number of HGD cases was an unexpected finding that may have clinical implications. The low expression level of ERBB2 in LGD was a novel finding and may indicate the role of ERBB2 in the early stages of Barrett carcinogenesis.

Molecular Testing

Molecular testing has been proposed to accurately detect and grade dysplasia and adenocarcinoma in patients with BE. Among these tests, FISH can be performed on endoscopic esophageal brushings, which can be used to sample a large diseased area of the esophagus. In a study by Brankley et al., various probe combinations were used to select a combination of FISH probes specific for identifying dysplasia and carcinoma in the setting of BE (Table 2). A total of 170 brushing specimens from 138 patients with BE were examined. Biopsy results were used as the gold standard. The authors found that probes to 8q24, 9p21, 17q11.2, and 20q13.2 detected LGD with sensitivity and specificity rates of 70% and 89%, respectively. The same probes were also able to identify HGD with sensitivity and specificity rates of 84% and 93%, respectively, and identify adenocarcinoma with sensitivity and specificity rates of 94% and 93%, respectively. Thus, these probes have been proposed for testing in prospective clinical trials.

Selaru et al. used global gene expression profiling to study BE and esophageal cancer using DNA microarrays. They examined 13 esophageal resections or biopsies, including 7 cases of BE and 6 cases of EAC, using a microarray platform containing 8,000 complementary DNA (cDNA) clones. Gene profiles were similar in all cases of BE, which clustered together and were different from the EAC cases (also clustered together). The researchers also differentiated adenocarcinoma from squamous cell carcinoma and signet ring cell carcinoma.

In another study, Helm et al analyzed microarrays so that they could obtain expression profiles from individual genes based on biopsy samples (9 cases of EAC/BE from which 3 cancers had arisen). To use as a reference point, the researchers pooled samples of BE from 6 patients with BE but without cancer or dysplasia. Their findings showed that increasing changes in gene expression were related to the progression from BE to EAC. The early loss of gene function that governs differentiation began prior to such histological change, whereas a gain in gene function was associated with the progression of invasiveness and remodeling.

Using DNA microarrays, Hao et al focused on the contributions of the stroma to the neoplastic process, studying the gene expression profiles of EAC or BE specimens obtained via biopsy and the associated normal duodenum and esophagus among 17 volunteers. The researchers found a unique expression profile for each tissue. Both EAC and BE had a unique set of 37 stromal genes distinct from those found in normal tissue. Collagen 5A2 and peristin were validated by in situ hybridization. No differences in gene expression were seen between long- and short-segment-type BE. The authors concluded that stromal gene changes precede transformation.

Ultrasensitive Complementary DNA Microarray Chip

In 2007, Ito et al introduced an ultrasensitive cDNA microarray chip for gene-expression profiling to be used in preoperative esophageal cancer biopsies that requires 0.1 to 0.01 µg of total ribonucleic acid (RNA). However, no RNA amplification is needed.

<table>
<thead>
<tr>
<th>Table 2. — FISH Probe Sets</th>
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<td><strong>Probe Set</strong></td>
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<td>II</td>
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*Formerly known as HER2/neu. CEP = centromere enumeration, FISH = fluorescence in situ hybridization, LSI = locus-specific indicator.

This method is highly sensitive. It might be suitable for clinical diagnosis because it discriminates noncancerous tissue from cancerous tissue and has accuracy, sensitivity, and specificity rates of 95.2%, 95.7%, and 94.7%, respectively.

MicroRNA

The expression of short, noncoding RNA influences genes by blocking the translation of target messenger RNAs or by quickening their degradation. RNA = ribonucleic acid. Reprinted from Curr Opin Pharmacol, 9(6), Kan T, Metzger SJ. MicroRNAs in Barrett's esophagus and esophageal adenocarcinoma, 727-732, Copyright 2009, with permission from Elsevier.

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Pathologists play a crucial role in the multidisciplinary care of patients with sarcoma.

Molecular Pathology of Soft-Tissue Neoplasms and Its Role in Clinical Practice
Evita B. Henderson-Jackson, MD, and Marilyn M. Bui, MD, PhD

Background: Soft-tissue neoplasms embody a histologically diverse group of mesenchymal tumors. Oftentimes the histopathological diagnosis of soft-tissue tumors is challenging due to overlapping pathological features.

Methods: We reviewed the current and most importantly known recurrent or tumor-specific genetic abnormalities involving soft-tissue tumors, focusing on how they are useful in working up differential diagnoses and the relevance of potentially targeted therapies.

Results: Molecular diagnostic tools have shown great advantage as an aid in the differentiation between different soft-tissue tumor entities, providing a potential avenue in the identification of novel therapeutic targets. Gastrointestinal stromal tumor is a well-known example of a soft-tissue tumor with a successful, molecularly driven treatment with response rates of more than 80% in stable disease and partial remission. Classifying soft-tissue neoplasms by their molecular genetic pathology has been considered as molecular testing becomes more integrated into various diagnostic and prognostic algorithms.

Conclusions: Molecular pathology provides a unique opportunity for pathologists to play a crucial role in the multidisciplinary care of patients with sarcoma. These opportunities include but are not limited to the appropriate triage of tissue for molecular testing and the integration of molecular testing results, with histological and immunohistochemical findings providing actionable information for the diagnosis, prognosis, and choice of therapeutic modality.

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Introduction
Benign and malignant soft-tissue neoplasms are rare and have diverse clinical and biological behavior. This heterogeneous group of mesenchymal tumors are diagnosed and classified using criteria from the World Health Organization (WHO); more than 100 benign and malignant soft-tissue tumor entities have been defined.1 These tumors are predominantly classified by their histogenesis and incorporated cytogenetic and molecular genetic information. In practice, pathologists use a morphological pattern recognition approach to analyze the histological information. The tumors can be arbitrarily divided into different morphological groups such as round cell, spindle, epi-
thelioid/polygonal, pleomorphic, adipocytic, myxoid, and giant cell, among others. Each group consists of a collection of pertinent differential diagnoses from various histogenesis. Immunohistochemistry (IHC) is an important ancillary tool to aid in diagnosis because it can provide information about tumor histogenesis.2

Cytogenetic and molecular genetic analyses of mesenchymal neoplasms have revealed 2 major groups: (1) soft-tissue neoplasms associated with complex karyotypes, and (2) soft-tissue neoplasms characterized by recurrent chromosomal structural abnormalities, gene amplification, mutations, or loss of heterozygosity. Although soft-tissue neoplasms with complex karyotypes do not demonstrate reproducible molecular alterations precluding the use of routine molecular testing for diagnostic purposes, the latter group can benefit from routine molecular testing to facilitate a definitive diagnosis.

With the continuous advancement in our understanding of the cytogenetic and molecular genetic pathology of soft-tissue neoplasms, pathologists evaluating soft-tissue tumors must rely on histopathology for diagnosis and integrate cytogenetic and molecular genetic information into diagnostic algorithms for accurate diagnoses. Fusion genes resulting from chromosomal rearrangements in soft-tissue neoplasms represent an important part of the pathologist’s diagnostic algorithm for tumor classification.3 Typically, chromosomal translocations and fusion genes are present in the early phase of disease and persist in disease progression, metastatic development, or both, demonstrating that molecular testing for these types of genetic alterations would be diagnostically useful. Furthermore, the identification of particular soft-tissue tumoral genetic aberrations is important for therapy.

In this article, we provide an overview of the important recurrent or tumor-specific molecular aberrations associated with soft-tissue tumors, their differential diagnoses, the role of molecular testing in the clinical management of soft-tissue tumors, and potentially relevant therapeutic targets.

Round Cell Tumors

Small round blue cell tumors encompass a group of primitive-appearing neoplasms difficult to diagnosis on histopathology alone. The differential diagnoses include a neoplasm of epithelial, mesenchymal, hematopoietic, or melanocytic origin. IHC may be helpful in narrowing the differential, as it is particularly effective for identifying carcinoma, neuroendocrine carcinoma, lymphoma, plasmacytoma, and melanoma. However, IHC is often ineffective in distinguishing between different round cell sarcomas.4 Most round blue cell tumors are associated with a tumor-specific chromosomal translocation, which is diagnostically valuable to pathologists.1,5,6

Ewing sarcoma, the second most common sarcoma of bone in children and young adults, occurs in extraskeletal sites in about 10% to 20% of cases.7,8 The histopathology and IHC profile may mimic poorly differentiated synovial sarcoma with round cell morphology.5,8,10 Molecular testing is often necessary to establish an accurate diagnosis. Ewing sarcoma is known to demonstrate recurrent translocations. t(11;22)(q24;q12) is detected in approximately 85% of Ewing sarcomas and results in the fusion of EWSR on chromosome 22 to a member of the ETS gene family of transcription factors, FLI1, on chromosome 11.11,12 Approximately 5% to 10% of Ewing sarcoma cases are associated with t(21;22)(q22;q12), which is a fusion of EWSR1 and ERG.13 Rare cases (< 1%) of Ewing sarcoma demonstrate FUS-ERG or FUS-PEV fusions, likely because the fused in sarcoma (FUS) protein amino-acid sequence is similar to Ewing sarcoma breakpoint region 1 (EWSR1) and is considered part of the tet methylcytosine dioxygenase (TET) family of proteins.14 The EWSR1 gene rearrangement is shared by numerous sarcomas, including Ewing sarcoma, myxoid liposarcoma, angiomatoid fibrous histiocytoma, myoepithelioma, myoepithelial carcinoma, mixed tumor of soft tissue, clear cell sarcoma of soft tissue, extraskeletal myxoid chondrosarcoma, malignant gastrointestinal neuroectodermal tumor (previously referred to as clear cell sarcoma-like gastrointestinal tumor), low-grade fibromyxoid sarcoma, and desmoplastic small round cell tumor.1,5,6,15 Recently, the WHO classification described a group of small round cell sarcomas with features similar to Ewing sarcoma (“Ewing-like” sarcoma) but classified the group as undifferentiated round cell sarcomas because the tumors either demonstrated rearrangements of EWSR1 with non-ETS gene partners or lacked rearrangement of EWSR1 or other TET family members.14,16-22 A small subset of these Ewing-like sarcomas (also known as undifferentiated round cell sarcomas) demonstrates CIC-DUX4 gene fusion resulting from t(4;19)(q35;q13) or t(10;19)(q26;q13) and is primarily described within the pediatric population.19,25-27 Moreover, the CIC-FOXO4 gene fusion, t(X;19)(q13;q13.3), has also been identified in 2 cases of Ewing-like sarcoma.26,27 In addition, an emerging group of Ewing-like sarcomas with the BCOR-CCNB3 fusion gene, arising from an X chromosome paracentric inversion, may represent a biologically distinct entity within undifferentiated round cell sarcomas.28-30 Simple reverse transcription polymerase chain reaction (RT-PCR) assay in conjunction with cyclin B3 (CCNB3) IHC can be useful in diagnosing these tumors. Whether these Ewing-like sarcoma cases should be classified as Ewing sarcoma or represent separate tumor types is unknown, but they have similar treatment.31 Further identification and clinical follow-up of Ewing-like sarcomas with
distinct gene rearrangements are warranted to evaluate patient outcomes.

Alveolar rhabdomyosarcoma (ARMS), often seen in adolescents and young adults, carries 2 specific chromosomal translocations.\(^{32}\) t(2;13)(q35;q14) occurs in approximately 60% of cases, whereas t(1;13) (p36;q14) occurs in a smaller subset.\(^{33,36}\) These translocations involve FOXO1A on chromosome 13q14 and either PAX3 (2q35) or PAX7 (1p36).\(^{35}\) No specific chromosomal abnormality has been described in embryonal rhabdomyosarcoma.\(^{37}\) Thus, the identification of t(2;15) or t(1;13) is diagnostically valuable and prognostically significant because the PAX-FOXO1A fusion status imparts an unfavorable outcome for children with rhabdomyosarcoma.\(^{3}\) A report of event-free survival and overall survival rates at 5 years correlated histopathological subtype with PAX-FOXO1A status and showed that fusion-negative ARMS (lacking a detectable fusion of PAX3 or PAX7 with FOXO1A) had an outcome similar to embryonal rhabdomyosarcoma and superior event-free survival rates compared with ARMS with either fusions of PAX3 or PAX7 with FOXO1A when given therapy designed for children with intermediate-risk rhabdomyosarcoma.\(^{38,39}\) These findings support the incorporation of PAX-FOXO1A fusion status into risk stratification and treatment.\(^{40}\)

Desmoplastic small round cell tumor primarily affects children and young adults and typically presents with widespread abdominal serosal involvement, exhibits polyphenotypic differentiation, and has a consistent association with t(11;22)(p13;q12) and fusion of EWSR1 and WT1.\(^{41-43}\) Because EWSR1 is shared by other sarcomas, identification of the partner gene is warranted for a specific diagnosis (Fig).

Extraskeletal myxoid chondrosarcoma (EMC) occurs in adults and, rarely, children, demonstrating no convincing evidence of cartilaginous differentiation; it is characterized by N\(_{\text{R4A3}}\) rearrangement.\(^{44-46}\) More than 90% of cases of EMC harbor either t(9;22) (q22;q12) or, less frequently, t(9;17)(q22;q11) resulting in the fusion of N\(_{\text{R4A3}}\) (9q22) to either EWSR1 (22q12) or TAF15 (17q12).\(^{47-50}\) IHC is not helpful in the diagnosis of EMC; therefore, molecular testing is essential. For example, EMC and mixed tumor of soft-tissue and myoepithelioma have overlapping histological and IHC features; thus, these entities present a diagnostic challenge. Considering the difference in therapies and outcomes of EMC and mixed tumor of soft-tissue and myoepithelioma, an accurate and definitive diagnosis is critical for the management of the condition. Because both tumors may show EWSR1 gene rearrangement and 75% of EMC cases and 45% of mixed tumor of soft-tissue and myoepithelioma cases exhibit this rearrangement,\(^{51}\) fluorescence in situ hybridization (FISH) testing of N\(_{\text{R4A3}}\) would be the ideal test to perform. Positive N\(_{\text{R4A3}}\) gene rearrangement, which represents a component of t(9;22) located on chromosome 9, would confirm the diagnosis of EMC.

This is because N\(_{\text{R4A3}}\) fusions are unique or specific for EMC and are not present in other sarcomas; in fact, N\(_{\text{R4A3}}\) fusions are present in more than 90% of EMC cases.\(^{1,5}\)

**Spindle Cell Tumors**

Clinically important, tumor-specific genetic abnormalities have been identified in spindle cell neoplasms. These genetic alterations are diagnostically helpful because the differential diagnoses of spindle cell neoplasms in soft tissue are diverse. Differentiation between tumor subtypes, such as monophasic synovial sarcoma, leiomyosarcoma, malignant peripheral nerve sheath tumor, fibrosarcoma, and gastrointestinal stromal tumor (GIST), may be challenging depending on specimen adequacy (quantity and quality), the immunostaining profile, and clinical presentation. Once the findings on the standard histopathological examination and IHC workup are inconclusive, molecular testing is a necessary adjunct. For example, synovial sarcoma can be difficult to discriminate from solitary fibrous tumor, malignant peripheral nerve sheath tumor, or sarcomatoid carcinoma. Given the ability to detect 95% of cases of synovial sarcoma through identification of its recurrent reciprocal t(X;18)(p11.2;q11.2), which fuses SYT (18q11) to 1 of the 3 homologous genes on Xp11 (SSX1, SSX2, or SSX4), molecular testing is essential for classification and treatment.\(^{52,53}\)

Dermatofibrosarcoma protuberans, a low-grade spindle cell neoplasm, harbors either supernumerary ring chromosomes or unbalanced derivatives of t(17;22)(q22;q13). Both aberrations contain a chimeric gene that fuses COL1A1 with PDGFB commonly identified by multiplex RT-PCR or, preferably, FISH.\(^{54-59}\) Typically, dermatofibrosarcoma protuberans is treated...
by wide local excision; however, in cases of unresectable and metastatic disease, targeted therapy with imatinib mesylate may be clinically useful. Molecular testing is important for identifying patients who may have a clinical response. A total of 10% to 15% of cases demonstrate fibrosarcomatous progression and may not show a response.¹

A rare malignant fibroblastic neoplasm, low-grade fibromyxoid sarcoma, consistently has either FUS-CREB3L2 gene fusion or, less frequently, FUS-CREB3L1 gene fusion and results in t(7;16) or t(11;16), respectively.⁶³,⁶⁴ A small number of low-grade fibromyxoid sarcomas harbor EWSR1-CREB3L1 gene fusion.⁶⁵ Sclerosing epithelioid fibrosarcoma (SEF), which is another rare malignant fibroblastic tumor with considerable morphological overlap with low-grade fibromyxoid sarcoma, harbors frequent EWSR1 gene rearrangements; a minority of cases exhibits FUS-CREB3L2 fusions.⁶⁶ Both low-grade fibromyxoid sarcoma and SEF show mucin 4 expression by IHC.⁶⁷ Thus, the presence of morphological features reminiscent of low-grade fibromyxoid sarcoma in SEFs as well as the presence of FUS rearrangements suggest that a possible relationship may exist between the 2 entities and that they are perhaps part of a disease spectrum.

Congenital or infantile fibrosarcoma, which is histologically similar to adult fibrosarcoma, has a distinctive t(12;15)(p13;q26) resulting in the ETV6-NTRK3 fusion. This neoplasm occurs in children within the first year of life and the histological features may mimic other pediatric spindle cell neoplasms, such as infantile fibromatosis and infantile myofibromatosis or myofibroma.³⁶⁶,⁶⁹ It is often difficult to cytogenetically identify the ETV6-NTRK3 fusion, so it is typically detected by FISH or PCR.⁷⁰,⁷¹

Congenital or infantile spindle cell rhabdomyosarcoma is another differential diagnosis in this group. This tumor typically lacks PAX3-FOXO1 and PAX7-FOXO1 fusions but exhibits recurrent NCOA2 rearrangements.⁷²

A breakthrough in the molecular pathology of solitary fibrous tumor (SFT) is the identification of the recurrent NAB2-STAT6 fusion by integrative sequencing.⁷³ NAB2-STAT6 has been established as the defining driver mutation of SFT. Fusion variants NAB2ex4-STAT6ex2/3 and NAB2ex6-STAT6ex16/17 have been identified; the former fusion occurs in pleuropulmonary SFTs and mostly exhibits a benign behavior, whereas the latter fusion occurs in deep-seated extrapleural SFTs and has more aggressive behavior.⁷⁴ STAT6 IHC can be a useful adjunct tool in the diagnosis of SFT, particularly in cases with aberrant morphology and limited material.⁷⁵,⁷⁶ Of note, STAT6 amplification has been described in a small subset of dedifferentiated liposarcomas resulting in STAT6 IHC expression, which can be a potential pitfall, particularly in retroperitoneal masses for which the differential diagnosis also includes SFT.⁷⁷

GIST is a mesenchymal tumor characterized by activating oncopgenic mutations rather than specific translocations or fusion genes and has a clinical spectrum that ranges from benign to malignant.¹⁷,⁷⁸ Most GISTs harbor a mutation in 1 of 3 sites: KIT exon 9, KIT exon 11, or PDGFRA exon 18.⁷⁹,⁸⁰ Tyrosine kinase inhibitors (eg, imatinib mesylate, sunitinib malate) have been used to successfully treat GISTs.⁸¹ Mutational analysis has become essential to evaluate patient prognosis, make treatment decisions, predict treatment response, and select appropriate dosages.⁷⁸,⁸²-⁸⁴ Most laboratories utilize PCR to amplify the most commonly mutated exons with subsequent direct sequencing analysis of the amplified exon.⁷⁹ Recently, BRAF V600E mutation was identified in patients with GIST lacking KIT and PDGFRA mutations.⁸⁵,⁸⁶ In addition, cases of GIST are typically associated with the Carney triad and Carney–Stratakis syndrome when they show mutations in SDH-related genes.⁸⁷,⁸⁸ These tumors have a distinct morphology.

**Lipomatous Tumors**

A few genetic alterations exist that involve lipomatous neoplasms and aid in discriminating tumor subtypes with similar morphological features. Myxoid/round cell liposarcoma may be challenging to diagnose when the differential diagnosis includes other myxoid neoplasms such as EMC or myxofibrosarcoma — this is particularly true on biopsy specimens. However, approximately 95% of cases of myxoid liposarcoma demonstrate the FUS-DDIT3 chimeric gene due to reciprocal t(12;16)(q13;p11).⁴³,⁸⁹,⁹⁰ Incorporating molecular testing with histopathology is useful in these cases because myxoid liposarcomas are sensitive to radiation therapy and select patients receive neoadjuvant therapy.⁹¹

Another diagnostic challenge is the differentiation between lipoma and atypical lipomatous tumor (ALT)/well-differentiated liposarcoma (WDL). Lipoma is a mass-forming tumor with mature-appearing fat; however, differently from normal fat, lipoma exhibits a HMGA2 translocation.⁹² The diagnostic atypical cells in fibrous septa can be scarce, the atypia may be cytologically subtle, or the ALT/WDL may demonstrate a lipoma-like morphological pattern. ALT/WDL is likely to recur and carries the risk of dedifferentiation, which results in a poor prognosis depending on the anatomical location. For clinical purposes, molecular testing is useful in discriminating between lipoma and ALT/WDL, and this is particularly true if clinicoradiological information is inconclusive. Furthermore, dedifferentiated liposarcoma with predominant, high-grade dedifferentiated areas may be difficult
to discriminate from other high-grade pleomorphic sarcomas. Molecular testing that identifies supernumerary ring chromosomes and/or giant-marker chromosomes corresponding to amplification of the 12q13-15 band support a diagnosis of ALT/WDL or dedifferentiated liposarcoma depending on histopathology.5,32 Of note, some lipomas have gains of the mouse double minute 2 homolog (MDM2) and some other mesenchymal tumors can have MDM2 amplification or expression (Table 1).23,93

**Tumor of Uncertain Histogenesis**

Tumors of uncertain histogenesis have a range of morphological features from round cells to spindle cells to epithelioid/polygonal cells. Some have characteristic translocations as well as unique and recognizable histopathological and clinical features. However, significant histopathological and IHC overlap exists between clear cell sarcoma (malignant melanoma of soft parts) and conventional melanoma. Clear cell sarcomas possess a recurrent EWSR1-ATF1 fusion in more than 90% of cases from a reciprocal t(12;22) (q13;q12).34,95 A related variant, t(2;22)(q32.3;q12), the EWSR-CREB1 fusion, has been reported in a small subset of clear cell sarcomas.96,97 Molecular testing is necessary to establish an unequivocal diagnosis of clear cell sarcoma.98 In addition, rare clear cell sarcomas have been reported to contain BRAF mutations, possibly representing a therapeutic target.99,100

Of note, alveolar soft-part sarcoma, which is a rare sarcoma composed of nests of tumor cells lined by sinusoidal vascular channels that may have eosinophilic, granular-to-clear cytoplasm, may be mistaken for renal cell carcinoma or perivascular epithelioid cell tumor.1 On IHC, alveolar soft-part sarcoma is negative for keratin, epithelial membrane antigen, and HMB-45. Molecular testing is diagnostically helpful to confirm the diagnosis. Alveolar soft-part sarcoma is defined by recurrent, unbalanced der(17)t(X;17) (p11;q25) involving the fusion of TFE3 (Xp11) and ASPSCR1 (17q25).101,102 Although ASPSCR1-TFE3 appears specific for alveolar soft-part sarcoma, this same gene fusion has been identified in a small subset of renal cell carcinomas.103,104

Soft-tissue angiofibroma is a relatively new histologic entity characterized as a benign, fibrovascular soft-tissue tumor of uncertain cellular origin. This tumor is characterized by an AHRR-NCOA2 fusion resulting from t(5;8) (p15;q13).105,106 The evaluation of NCOA2 rearrangements using FISH is a practical method to confirm a diagnosis of soft-tissue angiofibroma.105

**Myxoid Tumors**

Myxoid tumors do not represent a standalone group of tumors per the WHO criteria.1 However, this type of tumor is commonly encountered in clinical practice. The tumor cells may have various morphological features and they produce abundant myxoid stroma. The common genetic change of this group of tumor is summarized in Table 2. Among this group, myxoinflammatory fibroblastic sarcoma exhibits t(1;10) involving TGFB3 and MGEA5.107,108

**Conclusions**

Soft-tissue tumors form a heterogeneous and complex group with diverse morphology and, at times, a non-specific immunohistochemical profile. The availability of tumor-specific genetic markers has modified the routine diagnostic workup of soft-tissue neoplasms. The cytogenetic and molecular identification of chromosomal translocations and their associated gene fusions, loss, and gains of specific chromosomal regions and activating or inactivating mutations of select oncogenes or tumor-suppressor genes have contributed to therapeutic and prognostic assessments and the classification of soft-tissue neoplasms. The integration of morphology, immunohistochemistry, and molecular pathology is essential for appropriate and accurate diagnostic algorithms. A better understanding of how to apply the molecular pathology of soft-tissue neoplasms will provide new roles for pathologists in the diagnostics and targeted therapy of these tumors.3,109

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**Table 1. — MDM2-Positive Soft-Tissue Tumors**

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>MDM2 by FISH</th>
<th>MDM2 by IHC</th>
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<tbody>
<tr>
<td>Lipoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atypical lipomatous tumor/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>well-differentiated liposarcoma</td>
<td>+</td>
<td>+ (nuclear)</td>
</tr>
<tr>
<td>Dedifferentiated liposarcoma</td>
<td>+</td>
<td>+ (diffuse, nuclear)</td>
</tr>
<tr>
<td>Intimal sarcoma</td>
<td>+</td>
<td>+ (&lt;70%)</td>
</tr>
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FISH = fluorescence in situ hybridization, IHC = immunohistochemistry, MDM2 = mouse double minute 2 homolog.

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**Table 2. — Genetics of Myxoid Sarcomas**

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Defect</th>
<th>Gene</th>
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<tr>
<td>Myxoma</td>
<td>Activating Gs-α mutations</td>
<td>GNAS</td>
</tr>
<tr>
<td>Low-grade fibromyxoid sarcoma/hyalinizing spindle cell tumor with giant rosettes</td>
<td>t(7;16)(q33;p11) t(11;16)(p11;q11) t(16;xx)(p11)</td>
<td>CREB3L2-FUS CREB3L1-FUS EWSR1-CREB3L1</td>
</tr>
<tr>
<td>Myxoid liposarcoma</td>
<td>t(12;16)(q13;p11) t(12;22)(q13;q12)</td>
<td>DDIT3-FUS DDIT3-EWSR1</td>
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<tr>
<td>Extraskeletal myxoid chondrosarcoma</td>
<td>t(9;22)(q22;q12) t(9;17)(q22;q11) t(9;15)(q22;q21) t(3;9)(q11;22)</td>
<td>NR4A3-EWSR1 NR4A3-TAF2N NR4A3-TCF12 NR4A3-TFG</td>
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<tr>
<td>Myxofibrosarcoma</td>
<td>None characteristic</td>
<td>None</td>
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<tr>
<td>Myxoinflammatory fibroblastic sarcoma</td>
<td>t(1;10)</td>
<td>TGFB3-MGEA5</td>
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References


Advances in \textit{EGFR} as a Predictive Marker in Lung Adenocarcinoma

Farah K. Khalil, MD, and Soner Altiok, MD, PhD

\textbf{Background:} Worldwide, lung cancer is the most common cause of mortality. Toxins from tobacco smoke are known to increase the risk of lung cancer; however, up to 15\% of lung cancer–related deaths in men and up to 50\% of lung cancer–related deaths in women occur in people who do not smoke. Despite the fact that chemotherapy generally provides a survival benefit for non–small-cell lung cancer, not every patient will respond to therapy and many experience therapy-related adverse events. Thus, predictive markers are used to determine which patients are more likely to respond to a given regimen.

\textbf{Methods:} We reviewed the current medical literature in English relating to predictive markers that may be positive, such as the presence of an activating \textit{EGFR} mutation.

\textbf{Results:} The advances in using \textit{EGFR} as a molecular predictive marker were summarized. This biomarker influences therapeutic response in patients with lung adenocarcinoma. Clinical evidence supporting its value is also reviewed.

\textbf{Conclusions:} The use of \textit{EGFR} as a predictive factor in lung adenocarcinoma may help target therapy to individual tumors to achieve the best likelihood for long-term survival and to avoid adverse events from medications unlikely to be effective.

\textbf{Survival and Response Rates}

Across all treatments and tumor stages, the 5-year overall survival (OS) rate for non–small-cell lung cancer (NSCLC) is 16\%.

Patients receiving induction chemotherapy with carboplatin, paclitaxel, and bevacizumab followed by concurrent chemotherapy with erlotinib, carboplatin, paclitaxel, and bevacizumab in addition to radiation and consolidation therapy with erlotinib and bevacizumab have objective response rates between 39\% and 60\% and median progression-free survival (PFS) of 10.2 months to 18.4 months.\textsuperscript{5} Response rates for patients with metastatic NSCLC provided treatment are 17\% to 37\%; median survival rates are 6.7 to 11.3 months; and 1- and 2-year survival rates are 31\% to 46\% and 9\% to 21\%, respectively.\textsuperscript{1,3-5} Time to progression averages 4 to 6 months and response rate to second-line therapy is 8\%.\textsuperscript{6}

\textbf{EGFR and Tyrosine Kinase Inhibitors}

The most promising and widely reported molecular predictive factor in NSCLC is \textit{EGFR}, which resides on
the short (p) arm of chromosome 7 at position 12; epidermal growth factor (EGF) was first identified by Cohen in 1962. When EGF binds to epidermal growth factor receptor (EGFR), the receptor dimerizes, autophosphorylates, and activates several pathways, including mitogen-activated protein kinase, Janus kinase 2/signal transducer and activator of transcription (STAT) 3, STAT5, and phosphatidylinositol 3-kinase (PI3K)/protein kinase B pathways, which lead to cell proliferation, metastasis, and migration while preventing apoptosis. Because the EGF normally favors growth and proliferation, mutations of its receptor render it constitutively active independent of ligand-binding, which may lead to a malignant phenotype (Fig 1). An increased EGFR copy number or overexpression may play a role in oncogenesis. For example, based on their mechanisms of action, EGFR monoclonal antibodies (eg, cetuximab, matuzumab, panitumumab), which bind the extracellular domain of EGFR and prevent ligand binding, may be beneficial to patients with tumors overexpressing surface EGFR, whereas tyrosine kinase inhibitors (TKIs) may benefit patients whose tumor proliferation and metastases are driven by EGFR autophosphorylation. Blocking the catalytic site of the receptor, which is responsible for activating downstream molecules, may prevent growth and proliferation and possibly favor apoptosis in cancer cells.

Erlotinib and gefitinib, which are first-generation reversible TKIs, target the catalytic domain of EGFR by competing with adenosine triphosphate. Compared with placebo, erlotinib has been shown in a randomized, placebo-controlled, phase 3 trial to provide significant survival benefit in unselected individuals with advanced-stage NSCLC who received prior treatment. The researchers also found that those with certain demographic characteristics responded better to erlotinib. Other studies have demonstrated that patients who are Japanese, women, and those with adenocarcinomas have high response rates to gefitinib, whereas tyrosine kinase inhibitors (TKIs) may benefit patients whose tumor proliferation and metastases are driven by EGFR autophosphorylation. Blocking the catalytic site of the receptor, which is responsible for activating downstream molecules, may prevent growth and proliferation and possibly favor apoptosis in cancer cells.

Fig 1. — EGFR involved in tumorigenesis of non–small-cell lung cancer. Akt = protein kinase B, EGF = epidermal growth factor, EGFR = epidermal growth factor receptor, MEK/MAPK, mitogen-activated protein kinase, PI3K = phosphatidylinositol 3-kinase, PTEN = phosphatase and tensin homolog, RAF = v-raf 1 murine leukemia viral oncogene homolog 1, src = proto-oncogene tyrosine-protein kinase Src.

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Molecular Predictors of Response to Tyrosine Kinase Inhibitors and Survival Rates

The different mutations identified in EGFR are in exons 19 (58%), 21 (36%), and 18 (6%). Approximately 90% of the mutations are either small deletions from exon 19 (codons 746–750) or substitution of arginine for leucine at position 858 in exon 21 (L858R). Another 3% are substitutions of a variety of amino acids in place of glycine at codon 719 (G719X) in exon 19, and 3% are in-frame insertions in exon 20. The overall incidence of EGFR mutations in NSCLC ranges from 12.1% to 49%, depending on the patient population. Tyrosine kinase domain mutations of EGFR are rare in tumors in non-lung locations.

The exon 19 deletions and L858R substitution are activating mutations that result in increased phosphorylation of EGFR independent of ligand stimulation. These mutant EGFRs have greater affinity for TKIs and are susceptible to tyrosine kinase inhibition in vitro. Both Ji et al and Politi et al demonstrated that expression of either the exon 19 deletion or the L858R substitution mutation in EGFR causes adenocarcinoma to develop in mice and that tumor regression takes place when either expression of the mutant gene is blocked or erlotinib was administered. Greulich et al obtained similar results in cell cultures and confirmed that these mutations result in constitutive ligand-independent receptor activation, which is further enhanced by stimulating the ligand.
The usefulness of the *EGFR* mutation status as a predictive factor for response to TKIs is determined through the results of clinical trials and retrospective studies. Lynch et al\(^{34}\) demonstrated that *EGFR* mutations are associated with a clinical response to gefitinib and showed that in vitro *EGFR* mutated tumors were more sensitive to gefitinib. No mutations were present in patients who did not respond to gefitinib.\(^{34}\) Both in vitro exon 19 deletion mutants and L858R mutants were more sensitive to gefitinib than tumors with wild-type *EGFR*.\(^{34}\) In addition, all 8 mutants Lynch et al\(^{34}\) identified were heterozygotes, indicating that these mutations act in a dominant manner, similar to other oncogenes. Paez et al\(^{23}\) confirmed these results, and Pao et al\(^{35}\) soon added that *EGFR* mutations can also be found in tumors sensitive to erlotinib. Since then, other studies have confirmed the association between *EGFR* mutation status and response to TKIs,\(^ {30-41}\) and a review by Mitsudomi et al\(^{12}\) who compiled data from 1,335 patients showed that *EGFR* TKI response was observed in 70% of *EGFR*-mutant tumors compared with 10% of *EGFR* wild-type tumors. In a phase 2 trial, *EGFR*-mutant tumors responded better to gefitinib than wild-type *EGFR* tumors.\(^ {38}\) Kalikaki et al\(^{12}\) demonstrated that patients with classic-activating *EGFR* mutations treated with gefitinib had a significantly longer median OS rate than those with wild-type *EGFR*; however, this relationship was not present in patients with other types of *EGFR* mutations. Significantly higher rates of objective tumor response to TKIs in *EGFR* mutants compared with wild-type *EGFR* have been seen in several studies,\(^ {19,20,40,43}\) whereas others have found no such relationship.\(^ {44}\)

Differential response to treatment between the different types of *EGFR* mutations have been observed by some authors. Mitsudomi et al\(^{12}\) reported that deletional mutations had higher response rates to gefitinib than other mutations — in particular, L858R. In addition, Hirsch et al\(^ {16}\) found that patients who had exon 19 deletions alone had significantly higher objective response rates than patients with exon 21 mutations. By contrast, Pao et al\(^ {35}\) found that in vitro wild-type *EGFR* and exon 19 deletion mutants similarly responded to gefitinib and erlotinib, whereas L858R mutants were approximately 10 times as sensitive.

Not every patient with an activating mutation of *EGFR* responds to TKIs.\(^ {46}\) Alternatively, some patients without *EGFR* mutations still respond to TKIs.\(^ {20,43,46}\) Yoshioka et al\(^ {47}\) published a phase 2 prospective study and determined that *EGFR* wild-type tumors have a modest response to erlotinib without irreversible toxicity. *EGFR* mutation status impacts treatment and has a concomitant impact on survival. Longer median survival (but not PFS) rates after first-line chemotherapy have been observed in individuals with *EGFR* mutations.\(^ {48}\) In addition, Hotta et al\(^ {36}\) found that OS and PFS rates following first-line chemotherapy are also significantly longer in patients with *EGFR*-mutant tumors compared with those with wild-type *EGFR* tumors. Moreover, use of gefitinib as first- or second-line therapy resulted in significantly longer PFS rates among patients with *EGFR*-mutant tumors than those treated with chemotherapy.\(^ {48}\) In a series conducted by Kosaka et al,\(^ {30}\) 397 individuals with *EGFR* mutations survived significantly longer than those with wild-type mutations; no survival difference was seen among those with an exon 19 deletion and L858R substitution.

### Molecular Alterations Conferring Resistance to Tyrosine Kinase Inhibitors

Although many tumors with *EGFR* mutations initially respond to TKIs, disease progression will typically occur within 6 to 12 months because of the selective survival of tumor cells whose acquired mutations have conferred resistance to these agents, as well as due to pre-existing–resistant subclones, the up regulation of other parallel signaling pathways, or the transformation of tumor cells to small cell carcinoma.\(^ {12,18,49,51}\) Even small clones of tumor cells containing a mutation conferring resistance to TKIs may have a significant clinical impact.\(^ {50,52}\) The most common secondary *EGFR* mutation leading to TKI resistance is the substitution of methionine in place of threonine at codon 790 (T790M) within exon 20.\(^ {18,49,55-55}\) After identifying the presence of the T790M point mutation in a patient initially sensitive to gefitinib but who later became resistant, Kobayashi et al\(^ {34}\) demonstrated in vitro that introducing the T790M mutation into previously gefitinib-sensitive NSCLC cells harboring either wild-type, an exon 19 deletion, or L858R-substitution *EGFR* genotypes induced gefitinib resistance. Pao et al\(^ {53}\) also demonstrated in vitro that NSCLC cells harboring the T790M mutation with otherwise wild-type or mutated *EGFR* (either exon 19 deletion or L858R substitution) did not respond to either erlotinib or gefitinib.

Structural analysis has also shown that the T790M point mutation of *EGFR* changes tyrosine in the catalytic domain to methionine, which is bulkier and prevents erlotinib — and presumably gefitinib — from binding.\(^ {18,26,54,56}\) The T790M mutation alters TKI binding and enhances the kinase activity of *EGFR* when coupled with the L858R mutation, thus offering cells with this mutation a survival advantage.\(^ {12,20,32,57}\) The T790M point mutation has been identified in approximately 50% of patients who had activating *EGFR* mutations and later acquired resistance to TKI TKIs.\(^ {18,50,55,55,58,59}\) Engleman et al\(^ {60}\) and Bonomi et al\(^ {19}\) demonstrated in vitro that malignant cells with activating mutations of *EGFR* initially sensitive to gefitinib become resistant after prolonged exposure to gefitinib, and they may
also acquire the T790M mutation in EGFR. Even so, the question arises as to whether TKI treatment induces the resistance mutation or if it results in the selection of resistant clones. Depending on the sensitivity rate of the assay used, the T790M mutation has been found in 0.5% to 3.6% of patients who have never been treated with TKIs. Laboratory studies and clinical validation also indicate that the presence of pre-TKI EGFR T790M could act as a negative predictor of PFS in patients with EGFR-activating mutations.

The exon 20 insertion mutation of EGFR (D770insNPG) has been shown by Greulich et al to confer erlotinib and gefitinib resistance in vitro; however, the response was obtained from the irreversible EGFR inhibitor CL-387,785. In another study, 2 women with adenocarcinoma harboring this mutation who never smoked both progressed despite gefitinib treatment. Intermediate sensitivity to erlotinib or gefitinib was observed in G719S of exon 18, and that sensitivity may be related to the specific mutation present.

Another missense mutation, D761Y in exon 19, has been reported to confer a milder degree of TKI resistance that may be clinically relevant. Greater resistance to gefitinib is produced when the T790M mutation occurs on the same DNA strand as the EGFR-activating mutation (L858R or exon 19 deletion), which is the most common scenario. This can be explained by understanding that the mutant allele alone will code for the mutant, constitutively active EGFR; likewise, binding of TKIs to these mutant, constitutively active EGFRs will be altered only if the resistance mutation has occurred on the mutant allele.

Low PTEN expression is an important reason for tumors becoming resistant to TKIs. PTEN normally functions to down-regulate the PI3K pathway (downstream of EGFR; it mediates growth, proliferation, and survival). Loss of PTEN leads to persistent activation of the PI3K pathway independent of EGFR signaling, making this proliferation signal unresponsive to the EGFR blockade. Among gefitinib-resistant cells lacking PTEN, Bianco et al demonstrated that the reconstitution of PTEN reversed EGFR-independent PI3K pathway activity and restored gefitinib sensitivity. Zhuang et al demonstrated that irradiating TKI-resistant tumors with low PTEN expression may help reverse the resistance by increasing PTEN expression levels. Although low PTEN expression may induce resistance to TKIs, a relationship may not exist between PTEN expression and rate of survival.

Some authors have reported acquired resistance to EGFR-resistant TKIs in tumors with MET amplification. It has also been suggested that MET activation (which may be induced by hepatocyte growth factor binding, overexpression, or structural alterations) may be used in place of MET amplification to determine sensitivity to TKIs. The results of an in vitro study by Rho et al suggested that sensitivity to TKIs was not associated with MET activation in the absence of MET amplification.

Methods to Overcome Acquired Resistance to Tyrosine Kinase Inhibitors

Although T790M makes cells resistant to gefitinib and erlotinib (which are reversible TKIs), the effect of irreversible TKIs (which covalently bind the EGFR kinase domain) is not changed by T790M. Irreversible TKIs include afatinib, dacomitinib, neratinib, pelitinib, canertinib, and CL-387,785. Irreversible EGFR inhibitors such as afatinib inhibit EGFR and downstream molecules more potently than gefitinib or erlotinib in cells harboring the T790M mutation, although not to the degree observed in cells without the T790M mutation.

Regales et al demonstrated in mice that tumors harboring the T790M TKI–resistance mutation had more shrinkage (and a complete response in most tumors) when treated with combination afatinib/cetuximab (an EGFR-specific antibody) when compared with other antitumor agents. This combination resulted in lower total and phosphorylated EGFR. Neither agent alone induced a complete response in any of the tumors in the study. Another study showed that CUDC-305, a heat-shock protein 90 inhibitor, had inhibited tumor growth in mouse xenograft models of NSCLC with EGFR T790M mutations. Blocking signaling molecules downstream of EGFR has the potential to overcome the resistance induced by various mutations that alter the binding of drugs to EGFR. Src is one such downstream signaling molecule vital to maintaining the malignant phenotype of EGFR-mutant cells. In vitro, Src inhibitors have been shown to prevent oncogenesis caused by EGFR mutations, suggesting that Src inhibitors may be of clinical benefit in TKI-resistant EGFR-mutated NSCLC. Faber et al demonstrated in vitro that blocking 2 major pathways downstream from EGFR resulted in tumor shrinkage, even in cell lines with various TKI-resistant mutations, including T790M. Afatinib and dacomitinib have shown sustained control of disease progression in patients with NSCLC who have primary or acquired TKI resistance. The LUX-Lung 1 phase 2/2B trial investigated afatinib compared with placebo in patients with NSCLC who were progressing following treatment with erlotinib or gefitinib and combination platinum-based chemotherapy. No significant improvement in OS rate was seen when compared with placebo. Individuals naive to EGFR-TKI treatment with stage 3B and stage 4 NSCLC...
who harbored activating \textit{EGFR} mutations were studied during the LUX-Lung 2 trial.\textsuperscript{51} These study volunteers were given once-daily dosing of afatinib (40 or 50 mg), and similar overall response rates were seen; however, more adverse events were seen among those given the higher dose.\textsuperscript{51} The phase 3 trial, LUX-Lung 3, randomized 345 individuals with \textit{EGFR} mutation-positive adenocarcinoma to either once-daily 40 mg afatinib or combination cisplatin/pemetrexed.\textsuperscript{71} In that trial, 72% of study volunteers were of Asian descent.\textsuperscript{71} The afatinib group showed a prolonged PFS rate (11.1 vs 6.9 months) compared with the cisplatin/pemetrexed group.\textsuperscript{71} Patients with common \textit{EGFR} mutations (eg, L858R, exon 19 deletion) had a maximum PFS rate of 13.6 months when treated with afatinib, whereas those assigned to chemotherapy had a maximum PFS rate of 6.9 months.\textsuperscript{51,72}

The effective afatinib dose has toxicity limitations that has necessitated continual studies, thus leading to third-generation, irreversible TKIs, such as rociletinib, AZD9291, and HM61713, which have shown profound and sustained regression in T790M-mutant–selective resistance in NSCLC.\textsuperscript{73,74} Preliminary reports have shown response rates of 58%, 64%, and 29% for rociletinib, AZD9291, and HM61713, respectively.\textsuperscript{75,76} AZD9291 does not have the same severe adverse events as those seen with first-generation TKIs because it does not specifically target wild-type \textit{EGFR}, it is associated with reduced rates of skin rash and diarrhea, and it has an overall response rate of 64% in patients positive for T790M.\textsuperscript{74} A recent trial showed that no dose-limiting toxicities were present at doses between 20 and 240 mg/day.\textsuperscript{75}

**Correlation of Histological Subtype of Adenocarcinoma With \textit{EGFR}-Mutation Status**

NSCLC and small cell carcinoma are 2 major categories of lung cancer based on histology and response to chemotherapy. The most common cell type of NSCLC is adenocarcinoma. The International Association for the Study of Lung Cancer, the American Thoracic Society, and the European Respiratory Society International proposed a new classification of lung adenocarcinoma in 2011.\textsuperscript{77} This international, multidisciplinary classification divided adenocarcinoma into histological subtypes based on survival data that indicated 5-year survival rates of 100% among patients with adenocarcinoma in situ (previously known as bronchioloalveolar carcinoma) and minimally invasive adenocarcinoma.\textsuperscript{77} Invasive adenocarcinoma subtypes include lepidic, acinar, papillary, micropapillary, and solid predominant patterns. Mucinous, colloid, fetal, and enteric histology have been described as variants of adenocarcinoma.\textsuperscript{77} Results from clinicopathological studies have identified a correlation between histological subtypes and the clinical outcome in which adenocarcinoma in situ and minimally invasive adenocarcinoma had excellent outcomes; lepidic, acinar, and papillary predominant adenocarcinoma had intermediate outcomes, whereas solid and micropapillary predominant and invasive mucinous adenocarcinoma had poor outcomes.\textsuperscript{78} Fig 2 illustrates invasive adenocarcinoma of the lung with a micropapillary growth pattern.

Reclassification studies of adenocarcinoma based on the recommendations from the International Association for the Study of Lung Cancer, the American Thoracic Society, and the European Respiratory Society International indicate that the lepidic pattern is associated with 44% of \textit{EGFR}-mutant lung cancers as compared with the acinar pattern, which is associated with 69% of \textit{EGFR}–wild-type lung cancers.\textsuperscript{77,79}

**Conclusions**

Of all the molecular alterations that may have predictive value in non–small-cell lung cancer, testing for \textit{EGFR} mutations is usually the first step in determining the course of adjuvant therapy. Activating \textit{EGFR} mutations and amplification predict the response of non–small-cell lung cancer to tyrosine kinase inhibitors. The use of these predictive factors help target therapy to individual tumors to achieve the best likelihood for long survival; they are also used to help avoid unnecessary adverse events related to therapies unlikely to have any effect. Studies showing post–tyrosine kinase inhibitor resistance due to mutations not detected prior to treatment impress the utility of reassessing tumors following treatment. Additional, large cohort studies are required to accurately determine the association between adenocarcinoma subtypes and \textit{EGFR}-mutation status. Mutational heterogeneity within a single tumor and morphological associations will most likely mandate the continual assessment of each type of cancer as it progresses from primary to metastatic and pretreatment to post-treatment status.
References


This article reviews the molecular classification of diffuse gliomas in the adult population.

Impending Impact of Molecular Pathology on Classifying Adult Diffuse Gliomas

Robert J. Macaulay, MD

Background: Progress in molecular oncology during the last decade has enabled investigators to more precisely define and group gliomas. The impacts of isocitrate dehydrogenase (IDH) mutation (mut) status and other molecular markers on the classification, prognostication, and management of diffuse gliomas are likely to be far-reaching.

Methods: Clinical experience and the medical literature were used to assess the current status of glioma categorization and the likely impact of the pending revision of the classification scheme of the World Health Organization (WHO).

Results: IDH-mut is a defining event in most adult fibrillary astrocytomas (FAs) and nearly all oligodendrogliomas (ODs). The IDH-mut status of most gliomas can be established by immunohistochemistry for the most common mutant of IDH1 (R132H). IDH wild-type (wt) diffuse gliomas include several familiar entities — in particular, glioblastoma (GBM) and most pediatric gliomas — as well as an assortment of less well-defined entities. The codeletion of 1p/19q distinguishes OD from FA, which, by contrast, shows frequent loss of the α-thalassemia/mental retardation syndrome X-linked protein. Mixed oligoastrocytomas are typically classifiable as either OD or FA using molecular testing.

Conclusions: The current practice of designating IDH-mut WHO grade 4 astrocytoma as secondary GBM will likely be discouraged, and primary or de novo GBM, which is always IDH-wt, may lose this qualification. Histologically, low- or intermediate-grade IDH-wt gliomas with molecular changes characteristic of GBM might justify the designation of GBM WHO grade 3. Mixed oligoastrocytoma is losing popularity as a diagnostic term because most cases will fall into either the FA or OD category. Distinguishing IDH-mut from IDH-wt tumors in clinical trials is likely to clarify sensitivity rates or tumor resistance among subgroups, thus suggesting opportunities for targeted therapy.
(OD), small cell glioblastoma (GBM) vs anaplastic OD, and ependymoma (EP) vs FA.

Many gliomas exhibit characteristic genetic, chromosomal, and/or biochemical signatures indicative of distinct pathophysiology, presaging a reproducible and reliable molecular overlay to histopathological diagnosis. Pertinent ancillary studies beyond histopathology may include immunohistochemical testing, chromosomal assessment, traditional and array-based genetics, epigenetic analysis, and ribonucleic acid (RNA) profiling (both messenger RNA [mRNA] and noncoding micro-RNA [miRNA]). The World Health Organization (WHO) classification of central nervous system tumors will likely incorporate these findings in its soon-to-be released update. Such an improved classification system may contribute to the improved clinical investigation of new and targeted therapies to combat the continued guarded outlook for most primary brain neoplasms. Of particular interest are the implications of isocitrate dehydrogenase (IDH) mutation (mut) status.

Two Groups of Gliomas

The traditional categorization of gliomas into their morphological subgroups (astrocytic, oligodendrogial, and ependymal) typically precedes textbook discussions of particular entities. This is likely to change. In a seminal, genome-wide study, Parsons et al documented IDH1 mutations in subsets of glial neoplasms. It was soon recognized that mutations of IDH1 are frequent in FAs, ODs, and MOAs, particularly at amino acid residue 132 (arginine), which is replaced by histidine in nearly 90% of tumors bearing an IDH1 mutation. This residue may also be replaced by cysteine, serine, or other amino acids, and mutations may rarely occur at other loci. IDH2 may also be affected, albeit at a different locus (residue 172). Mutations of other IDH isoforms have not been reported to accompany gliomagenesis. Thus, the finding of an IDH1 or IDH2 mutation can be termed IDH-mut, and the absence of a mutation in either gene is designated as IDH wild type (wt). Acute myeloid leukemia, chondrosarcoma, and giant cell tumor of the bone may also exhibit IDH mutations.

Accumulation of 2-Hydroxyglutarate

Early events in developing neoplasms involve the acquisition of aberrations in proliferation-related intracellular signaling pathways. IDH normally participates in the oxidative decarboxylation of isocitrate to α-ketoglutarate. The mutant form lacks this important enzymatic property and instead generates an abundance of 2-hydroxyglutarate (2HG). Excess 2HG interferes with chromatin-modifying activity, thus leading to global DNA hypermethylation and the glioma-CpG island methylation phenotype, which is linked to tumorigenesis. Therefore, 2HG can be designated as an oncometabolite. Because IDH alterations are fundamental to pathobiology, disease progression, diagnosis, response to adjuvant treatment, and clinical outcome, the classification of gliomas should first acknowledge IDH-mut vs IDH-wt status. This approach would solidify the analysis of subtypes of IDH-mut tumors, allowing more objective classification of IDH-wt entities, and highlight the molecular distinctions between pediatric and adult tumors exhibiting similar morphological features.

Common Morphological Entities

The 3 morphological entities that exhibit IDH-mut are FA (grades 2–4), OD (grades 2 and 3), and MOA (grade 2 or 3). All other brain tumors are IDH-wt, including most pediatric cases, pilocytic astrocytoma of any age, pleomorphic xanthoastrocytoma, subependymal giant cell astrocytoma, EP, medulloblastoma, and meningioma. IDH-wt diffuse astrocytomas, including de novo GBM, gliomatoses cerebri, thalamic, and brainstem astrocytoma, harbor a variety of genetic changes, and they should be regarded as distinct from their IDH-mut counterparts despite being morphologically indistinguishable. Conversely, cases of OD are almost always IDH-mut. Rare IDH-wt exceptions, including most pediatric cases, should be segregated within this diagnostic category until their pathogenesis is resolved. Morphologically MOAs are frequently IDH-mut and subsequently classifiable as either FA or OD following further molecular testing. The appropriate pathological diagnosis for the rare IDH-wt MOA remains unsettled; however, such tumors should still be segregated from the IDH-mut categories.

Immunohistochemistry

Once assigned to the diffuse glioma category by routine pathological assessment, testing for the IDH1 R132H mutation may be easily conducted using a commercially available antibody test that recognizes the altered epitope. A positive result has high sensitivity and specificity rates for IDH-mut R132H, although reports exist of cross-reactivity with R132L and R132M mutations. A total of 10% to 15% of IDH-mut cases are immunonegative, and such cases can be sent for direct sequencing or hotspot analysis for both IDH1 and IDH2. IDH1 R132C is the most common immunonegative mutation.

IDH1/2 Mutations Account for Most Fibrillary Astrocytomas

A patient with low-grade astrocytoma typically presents with new-onset seizures, headaches, focal neurological deficits, or all of these symptoms. Neuroimaging typically shows a hemispheric hypointense, nonenhancing mass that — if followed without sur-
Altered biology — may show minimal growth in the early stages. If magnetic resonance spectroscopy reveals a peak attributable to the accumulation of 2HG, then that result strongly predicts IDH-mut and is correlated with improved outcomes. Histopathology shows an astrocytic proliferation exhibiting coarse fibrillary processes and sufficient nuclear irregularity and hyperchromatism to justify designation as a neoplasm (Fig A–C). Approximately 70% of these tumors will exhibit IDH-mut (Fig D) as well as other changes. The gradual progression to high-grade astrocytoma is typical, although the time to progression is unpredictable. When WHO grade 4 features are documented, the designation of secondary glioblastoma has found favor. (Whether this term is appropriate, even at first diagnosis, is discussed below.) Cases with features reminiscent of oligodendroglioma (small round monomorphic cells with perinuclear haloes) may prompt a preliminary diagnosis of MOA or the more generic diffuse glioma (not otherwise specified) until further testing resolves the diagnosis.

**TP53 Mutations Common in IDH-mut Astrocytomas**

Mutations in TP53 have been recognized in astrocytoma, ranging from 30% to 50% of studied cases, and alterations in related genes are frequently detected in all grades of diffuse astrocytomas. Interactions between a p53 alteration and IDH-mut in the earliest stages of tumor initiation have been suggested by the predilection for the uncommon R132C mutation to occur in Li–Fraumeni syndrome (germline mutation of p53 with tumor predisposition). Nuclear p53 overexpression is common in astrocytoma, is readily detected with immunohistochemistry (Fig E), and often correlates with TP53 mutation; however, as a diagnostic or prognostic marker, its utility is limited, and it has been supplanted by IDH, α thalassemia/mental retardation syndrome X-linked (ATRX), and 1p/19q testing.

**ATRX Loss Characterizes IDH-mut Astrocytomas**

Mutations in the telomere maintenance protein ATRX appear to distinguish IDH-mut FA from tumors that lack this change — in particular, OD. Absence of ATRX protein (Fig F) by immunohistochemistry is evident in 25% to 30% of adult and pediatric high- and low-grade astrocytomas. Loss of ATRX expression and IDH-mut typifies more than 95% of adult diffuse astrocytomas, and it is almost mutually exclusive with 1p/19q codeletion. Classification based on IDH-mut status and ATRX expression will likely be a key recommendation in the next WHO classification update.

**WHO Grade 4 IDH-mut Fibrillary Astrocytoma**

WHO grade 4 FA with an IDH-mut accounts for fewer than 10% of WHO grade 4 gliomas, typically prompting the designation of secondary GBM, however, the weight of evidence, both molecular and clinical, suggests that this term is outdated. It is likely that IDH-mut WHO grade 4 astrocytoma arises from a different precursor pool than IDH-wt GBM. IDH-mut gliomas follow a distinct molecular trajectory from IDH-wt tumors. The ambiguity of the term “primary GBM” has been emphasized by the occasional de novo presentation of an IDH-mut WHO grade 4 astrocytoma or conversely by IDH-wt low-grade glioma progressing quickly to GBM. In addition, compared with those with IDH-wt GBM, patients with IDH-mut WHO grade 4 FA are younger and have longer survival times. Therefore, it is clear that IDH1-mut FAs — whatever the grade — and IDH1-wt gliomas are different diseases. Because GBM is a familiar term for pathologists and clinicians, it will likely be retained, but this term may be restricted to morphologically appropriate gliomas.
that lack IDH-mut. It will be interesting to determine whether the classic histopathological WHO grading system developed for diffuse astrocytoma when IDH status was not being tested would require modification for IDH-mut astrocytomas.

Characterizing Oligodendroglioma
The classic OD appearance of small round cells arranged in honeycomb-like nests with “chicken wire”-like coarse, branching vasculature, microcalcifications, and perinuclear haloes has withstood the scrutiny of advancing technologies. 1p/19q codeletion is prognostic, predictive, and diagnostic. In addition, more than 90% of 1p-/19q- ODs are also IDH-mut. Among IDH-mut gliomas, mutations in the promoter of TERT are confined to OD; conversely, OD lacks ATRX mutations. For practical purposes, grading of OD is restricted to grades 2 and 3 because patient outcomes are considerably better for OD than for IDH-wt gliomas. Surgical cases with ambiguous features can be initially designated as MOA or simply as diffuse glioma. Support is growing for the notion that most MOAs can be placed into either the FA or OD category using IDH status, ATRX expression, and 1p/19q codeletion testing, and that a genuine mixture of the 2 is a rare occurrence.

Diffuse IDH-wt Gliomas in Adults
About 30% of WHO low-grade adult diffuse astrocytomas and fewer than 10% of morphologically diagnosed OD are IDH-wt; by contrast, the majority of newly encountered GBM is IDH-wt. Among the IDH-wt low-grade astrocytoma group, some of these cases harbor chromosomal and molecular alterations, thus aligning them with de novo GBM. The remaining cases possess a variety of genetic and biochemical alterations, some of which are more akin to pediatric gliomas, particularly BRAF alterations and histone modifications, facilitating further subclassification.

IDH-wt Glioblastoma
The most common primary brain tumor, GBM, is also the most lethal. Histologically, GBM is populated by anaplastic astrocytes with mitotic activity, glomeruloid vascular proliferation, and geographic necrosis with (or without) peripheral pseudopalisading. The molecular landscape of GBM is complex and likely accommodates several distinct entities, as well as considerable intratumoral heterogeneity and overlap. Most GBMs arise rapidly and are clinically designated as being primary or de novo, whereas individuals with a lower-grade IDH-mut astrocytoma may progress to grade 4 (secondary GBM). Future classification may eliminate the “secondary” GBM designation because progression-free and overall survival rates as well as responses to adjuvant chemotherapy or radiotherapy are improved. If IDH status is unknown, then array-based genetic testing for IDH1 and IDH2 can be considered in patients for whom an increased likelihood of a positive result exists, particularly for those tumors that exhibit features such as oligodendrogial morphology or loss of ATRX expression.

Complex Molecular Signature of IDH-wt GBM
Among the myriad molecular alterations in GBM, EGFR mutations, deletions, and protein overexpression are common. Clinical trials targeting the epidermal growth factor receptor VIII mutant form, in which exons 2 to 7 are lost, have shown promise, but detecting the mutation, either by immunohistochemistry, reverse transcription–polymerase chain reaction, or multiplex ligation-dependent probe amplification, is of dubious significance in multivariate models. The significance of the loss of PTEN is unsettled in GBM, but it may be a poor prognostic indicator in diffuse gliomas, particularly in tumors lacking 1p/19q codeletion. TERT promoter mutations are detectable in about one-half of IDH-wt GBMs and only those lacking ATRX mutation; TERT promoter mutations in IDH-mut tumors are restricted to OD. Other changes, such as p16, NF, Rb, or CDKN2A, have yet to achieve diagnostic or therapeutic significance. Therefore, the molecular pathogenesis of sporadic GBM in adults remains uncertain. Sophisticated expression profiling, which allows grouping into proneural, neural, mesenchymal, and classical phenotypes, has yet to reach the point of diagnostic utility, possibly due to considerable intratumoral heterogeneity.

Intraoperative Diagnosis: High-Grade Glioma
A pathologist handling an aggressive neoplasm with ring enhancement and central necrosis may provide an intraoperative diagnosis of GBM if morphologically appropriate. However, IDH-mut FA may undergo anaplastic transformation following disease progression, or it may have histological features similar to IDH-wt GBM at presentation; alternatively, OD may acquire mitotic activity, vascular proliferation, and necrosis and yet still follow a clinical course more consistent with WHO grade 3. IDH-mut tumors maintain their molecular signatures over time and subsequent resections, and they have improved outcomes relative to IDH-wt GBM with similar grading features. Because these cases are morphologically indistinguishable on intraoperative smears and frozen sections, an intraoperative diagnosis of high-grade glioma for practical purposes would ensure the appropriate handling of diagnostic material. The final diagnosis could range from FA grades 3 or
4, OD grade 3, EP grade 3, GBM, or primitive neuroectodermal tumor/medulloblastoma (depending on location). It remains to be determined whether the preoperative demonstration of a 2-hydroxyglutarate peak on magnetic resonance spectroscopy is sufficient evidence of IDH-mut or conversely whether its absence is sufficient to imply IDH-wt.52

Glioblastoma WHO Grade 3

Considerable interest exists in the biology and clinical behavior of low- or intermediate-grade diffuse astrocytic IDH-wt neoplasms in adults. Some examples that share molecular features with GBM can be considered as being “missed” GBM due to undersampling. Other patients may present during the early phase of tumor progression and before florid mitotic activity, vascular proliferation, and necrosis have developed; biopsy findings may indicate FA WHO grade 2 or 3.40 The tumors with molecular findings of GBM will more rapidly advance than their IDH-mut counterparts, although their overall outcome is still better than GBM.51 In the future, such examples may be categorized as GBM WHO grade 3, and the designation of FA WHO grade 3 might be reserved for IDH-mut cases.

IDH-mut Is Uncommon in Pediatric Diffuse Glioma

IDH-mut glial neoplasms do not manifest before adolescence41; conceptually, this can be attributed to a slow accumulation of 2HG and a delayed development of the glioma-CpG island methylation phenotype. Molecular alterations predominantly observed in children and young adults correlate with patient age and tumor location.27,62 Recent studies have emphasized the prognostic significance of detecting the mutated form of histone 3 and will likely lead to its routine testing in midline pediatric high-grade astrocytoma.5,6,64 By contrast to the tumors seen in adults, pediatric OD is rarely accompanied by IDH-mut or 1p/19q codeletion.25,65 This indicates at least 2 forms of OD: the adult IDH-mut and pediatric IDH-wt types. Whether rare IDH-mut pediatric diffuse gliomas represent an early occurrence of their adult counterparts has not yet been established.61,66

Conclusions

Isocitrate dehydrogenase (IDH) mutation (mut) is an early and likely initiating event in the development of many gliomas. This provides an opportunity to more precisely classify the 2 IDH-mut tumor types, fibrillary astrocytoma and oligodendroglioma. Distinguishing IDH-mut from IDH wild-type (wt) tumors may improve our understanding of tumor biology, prognostication, patient stratification in clinical trials, and the selection of potential therapeutic targets.67 Failure to appreciate this fundamental dichotomy from the outset of study design risks a misinterpretation of results; for example, a comparison of micro-ribonucleic acid (RNA) in low- compared with high-grade gliomas may be better interpreted as comparing IDH-mut vs IDH-wt tumors.3,8 By contrast, a trial of the intratumoral heterogeneity of messenger RNA expression identified IDH-wt status as a fundamental study inclusion criterion.5

Glioblastoma remains an essential diagnostic category and will likely be subdivided on a molecular basis, but, ideally, it would exclude IDH-mut tumors. The diagnostic term secondary glioblastoma might be replaced with astrocytoma World Health Organization (WHO) grade 4 and restricted to IDH-mut tumors. The diagnostic term de novo glioblastoma could then be shortened to glioblastoma WHO grade 4 and restricted to IDH-wt tumors. Histologically lower-grade neoplasms of evidently similar biology may qualify for the designation of glioblastoma WHO grade 3 if they possess the molecular signature of glioblastoma but lack vascular proliferation and necrosis.

References


Overexpression of Vascular Endothelial Growth Factor A in Invasive Micropapillary Colorectal Carcinoma

Marilin Rosa, MD, Maisoun Abdelbaqi, MD, Katherine M. Bui, Aejaz Nasir, MD, MPhil, Marilyn M. Bui, MD, PhD, David Shibata, MD, and Domenico Coppola, MD

Background: Invasive micropapillary carcinoma (IMPC) is a rare variant of colorectal cancer with an adverse prognosis. “Retraction artifact” around tumor cells is a feature of IMPC. The aim of this study was to assess the nature of the retractions around the tumor cells and to describe the histopathological features of a group of 18 cases of IMPC.

Methods: A pathology review of 128 consecutive colorectal cancers identified 18 cases of histologically proven IMPC using 5% of the total tumor volume comprised of a micropapillary component as the diagnostic criterion. Immunostains for D2-40, CD31, CD34, vascular endothelial growth factor A (VEGF-A), and mucin 1 (MUC-1) were performed using the avidin-biotin complex method.

Results: Cases of IMPC were characterized by pseudomicropapillae surrounded by lacunar-like clear spaces. These structures exhibited the inside-out growth pattern as highlighted by MUC-1 staining. The lining of the lacunar spaces was immunoreactive to CD31 but not CD34 or D2-40, indicating that they are neovascular structures. Furthermore, the tumor cells strongly and diffusely expressed VEGF-A.

Conclusions: The strong coexpression of VEGF-A and CD31 suggests a prominent role of neoangiogenesis in these tumors.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the third leading cause of cancer death in both men and women in the United States. Invasive micropapillary carcinoma (IMPC) has recently been described as a rare variant of CRC with a poor prognosis independently of stage. Although a focal micropapillary pattern can be seen in conventional cases of CRC, the diagnosis of the micropapillary variant of CRC requires that at least 5% of the total tumor volume has a micropapillary component. The proportion of the micropapillary component has also been suggested to have an important predictive factor for nodal metastasis, but this finding has not been consistent.

IMPC has been recognized and studied at other anatomical locations such as the ovary, breast, urinary
bladder, salivary glands, and lung.\textsuperscript{6-10} In its breast counterpart, IMPC shows a tendency to invade lymphatic vessels and to spread to regional lymph nodes. Whether the lacunar-like spaces surrounding the micropapillary structures are true vascular channels or “retraction artifacts,” as is commonly considered, remains a subject of debate.\textsuperscript{11,12} Some published studies suggest that retraction clefts are likely the result of an altered tumor–stromal interaction.\textsuperscript{11,13} In addition, these retraction clefts may be potential spaces that represent prelymphatic spaces involved in the facilitation of the initial lymphatic invasion and may undergo endothelialization under the influence of growth factors secreted by tumor cells.\textsuperscript{11}

The histopathological, immunohistochemical, and molecular features of colorectal IMPC have been previously defined.\textsuperscript{1,5} In this study, we review a single institution series of colorectal IMPC cases and characterized their vascular immunohistochemical phenotype to understand whether the presence of retraction clefts correlates with lymphangiogenesis and, thus, is a factor facilitating metastatic tumor spread.

**Methods**

**Participants**

This study was carried out in accordance with a research protocol approved by the Institutional Review Board at the H. Lee Moffitt Cancer Center & Research Institute and the University of South Florida in Tampa, Florida. A total of 128 consecutive colon resections performed at the Moffitt Cancer Center during a 5-year period from 2005 to 2010 were retrospectively selected from the anatomical pathology files. Two pathologists (DC and MA) reviewed the pathology reports and the hematoxylin and eosin–stained microscopic slides of each tumor to confirm the diagnosis and to select a representative block for immunohistochemical studies. As in previously published studies, tumors were classified as IMPC when at least 5% of the tumor was comprised of a micropapillary component.\textsuperscript{2,5} In case of a discrepancy, the slides were reviewed again by both pathologists at a multiheaded microscope to reach a diagnostic consensus. Collected clinico-pathological variables included tumor size, grade, stage, histological type, and the presence of nodal metastases, distant metastases, or both.

**Immunohistochemistry**

Immunohistochemical analysis was performed on 5-µm unstained sections from formalin-fixed, paraffin-embedded representative tumor blocks with an autostainer using monoclonal antibodies against CD31, CD34, D2-40, vascular endothelial growth factor A (VEGF-A), and mucin 1 (MUC-1). All stains were run with appropriate controls and stained in accordance with the manufacturer’s recommended protocol. Results were considered positive when cytoplasmic staining, membranous staining, or both were present in any percentage of the cells of interest and considered negative when there was a complete lack of staining.

**Results**

Our review identified a total of 18 (14% of consecutive colorectal carcinomas) bona fide cases of IMPC. The age of the patients ranged between 34 and 93 years (mean, 52.3 years). Twelve patients were men and 6 patients were women. Eight tumors were located in the right colon (2 in the cecum), 4 in the left colon, 4 in the rectum, 1 at the splenic flexure, and 1 in the terminal ileum. The tumor size ranged from 2.0 to 6.1 cm (mean, 4.0 cm). Twelve tumors were diagnosed as moderately differentiated and 6 were poorly differentiated. The pathological stages of the tumors were as follows: 4 carcinomas were stage 1, 3 were stage 2A, 2 were stage 3B, 8 were stage 3C, and 1 was stage 4. Three patients received preoperative chemotherapy (Table).

<table>
<thead>
<tr>
<th>Location</th>
<th>Size (cm)</th>
<th>Grade</th>
<th>% of Micropapillary Component</th>
<th>No. of LNs</th>
<th>Morphology of LN Metastasis</th>
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</thead>
<tbody>
<tr>
<td>Ascending colon</td>
<td>4.5</td>
<td>3</td>
<td>90</td>
<td>0/15</td>
<td>NA</td>
</tr>
<tr>
<td>Ileocecal valve</td>
<td>4.3</td>
<td>2</td>
<td>10</td>
<td>1/11</td>
<td>Micropapillary</td>
</tr>
<tr>
<td>Right colon</td>
<td>3.5</td>
<td>3</td>
<td>10</td>
<td>3/26</td>
<td>1/3 micropapillary</td>
</tr>
<tr>
<td>Sigmoid colon</td>
<td>5.0</td>
<td>2</td>
<td>80</td>
<td>14/20</td>
<td>9/14 micropapillary</td>
</tr>
<tr>
<td>Rectum</td>
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<td>2</td>
<td>10</td>
<td>2/31</td>
<td>Micropapillary</td>
</tr>
<tr>
<td>Rectum</td>
<td>6.0</td>
<td>2</td>
<td>20</td>
<td>7/16</td>
<td>Micropapillary</td>
</tr>
<tr>
<td>Terminal ileum</td>
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<td>2</td>
<td>30</td>
<td>13/34</td>
<td>Slides not available</td>
</tr>
<tr>
<td>Rectum</td>
<td>3.2</td>
<td>3</td>
<td>10</td>
<td>0/23</td>
<td>NA</td>
</tr>
<tr>
<td>Right colon</td>
<td>4.0</td>
<td>3</td>
<td>10</td>
<td>18/18</td>
<td>3/18 micropapillary</td>
</tr>
<tr>
<td>Rectum</td>
<td>2.6</td>
<td>2</td>
<td>10</td>
<td>0/24</td>
<td>NA</td>
</tr>
<tr>
<td>Cecum</td>
<td>3.5</td>
<td>2</td>
<td>10</td>
<td>4/5</td>
<td>Micropapillary</td>
</tr>
<tr>
<td>Cecum</td>
<td>4.5</td>
<td>2</td>
<td>10</td>
<td>7/46</td>
<td>5/12 micropapillary</td>
</tr>
<tr>
<td>Ileocecal valve</td>
<td>6.5</td>
<td>3</td>
<td>50</td>
<td>15/49</td>
<td>13/15 micropapillary</td>
</tr>
<tr>
<td>Sigmoid colon</td>
<td>4.0</td>
<td>2</td>
<td>30</td>
<td>0/18</td>
<td>NA</td>
</tr>
<tr>
<td>Sigmoid colon</td>
<td>2.5</td>
<td>2</td>
<td>60</td>
<td>3/13</td>
<td>No micropapillary</td>
</tr>
<tr>
<td>Splenic flexure</td>
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<td>3</td>
<td>90</td>
<td>0/15</td>
<td>NA</td>
</tr>
<tr>
<td>Right colon</td>
<td>2.1</td>
<td>2</td>
<td>40</td>
<td>7/13</td>
<td>Micropapillary</td>
</tr>
<tr>
<td>Left colon</td>
<td>2.0</td>
<td>2</td>
<td>20</td>
<td>1/15</td>
<td>Micropapillary</td>
</tr>
</tbody>
</table>

LN = lymph node, NA = not applicable.
Histologically, all micropapillary areas exhibited the characteristically features of IMPC as previously described.\textsuperscript{2-5} Tumors were composed of clusters of cells growing in a micropapillary (or pseudomicropapillary) pattern surrounded by lacunar-like clear spaces lined by elongated endothelial-like cells (Fig 1A). Fibrovascular cores were not identified (Fig 1B). Tumor cells were columnar to polygonal and had a moderate amount of eosinophilic cytoplasm and conspicuous nucleoli. Characteristically, the micropapillae exhibited a rotation of cell polarization known as an “inside-out” growth pattern, or “reverse cell polarity,” which was also highlighted by MUC-1 immunostaining (Fig 1C). Collections of neutrophils were seen in some cases to infiltrate the micropapillae and occasionally to spill into the lacunar-like spaces (Fig 1D). In addition, the micropapillary features represented at least 10% of the tumors in all of our cases. The transition between conventional adenocarcinoma and IMPC was abrupt, and, in general, the IMPC component was identified at the advancing, infiltrating edge of the tumors.

Immunohistochemically, we observed a strong and diffuse expression of VEGF-A in the tumor cells within the lacunar-like spaces (Fig 2A). With regard to the vascular markers, CD31 — but not CD34 — positivity highlighted the cells lining the clear spaces surrounding the micropapillae, thus suggesting that they are immature vessels (Fig 2B and C). D2-40 also failed to stain this lining, suggesting that the peritumoral spaces are not lymphatic (Fig 2D).

**Discussion**

IMPC is an unusual and aggressive variant of colorectal adenocarcinoma commonly associated with lymphovascular invasion, lymph node metastasis, and poor clinical outcome.\textsuperscript{2-5} In our retrospective review of 128 cases of CRC, we identified 18 cases of IMPC (approximately 14%), similar to what was reported by Haupt et al\textsuperscript{4} (19% of 178 cases of CRC with IMPC-like features). Histologically, our cases also exhibited similar features to those previously described — in particular, the presence of micropapillae without a fibrovascular core surrounded by a clear lacunar space.\textsuperscript{2-5}

The “micropapillary” appearance, which was first described in tumors of the breast,\textsuperscript{10,11} has been attributed to a peculiar rotation of cell polarization seen in these tumors and is described as an inside-out growth pattern. This characteristic feature has been supported by electron microscopy studies, which have shown that the microvilli of the cellular surface are at the stromal–epithelial interface and not toward the lumen of the neoplastic gland.\textsuperscript{2} This inside-out pattern is better observed with MUC-1 immunostain\textsuperscript{2} and has been linked to stromal and vascular invasion.\textsuperscript{3-5}

Metastasis to local lymph nodes via the lymphat-
ly identified in this tumoral area may explain this early metastatic and local progression. In our series, of the removed 392 lymph nodes, 95 were positive for metastatic disease; the metastasis was of micropapillary morphology in 53 lymph nodes. Although the percentage of the micropapillary component did not appear to correlate with the presence or number of positive lymph nodes, a slight increase of papillary morphology was seen in the metastatic tumors (56% of lymph node metastases were of micropapillary morphology; see Table).

To explore the role of peritumoral spaces in angiogenesis, tumor progression, and metastatic spread of IMPC of a colorectal origin, we studied a panel of vascular markers (CD31, CD34, D2-40) and VEGF-A. CD31, also known as platelet-endothelial cell adhesion molecule 1 (PECAM-1), is a 130k-Da transmembrane glycoprotein expressed at high levels on early and mature endothelial cells, platelets, and most leukocyte subpopulations. In addition to its role in the adhesion and transmigration of inflammatory cells, CD31 has an important role in angiogenesis.14-17 DeLisser et al15 showed that PECAM-1 is involved in angiogenesis and suggested that endothelial cell–cell adhesion molecules are important in the formation of new vessels. PECAM-1 also plays a complex role in tumor-induced angiogenesis in which neovascularization arises from the interactions between multiple stimulatory factors (eg, VEGF, basic fibroblast growth factor, interleukin 8, angiogenin).16 CD31 is involved in the regulation of hematopoietic progenitor cell compartmentalization between the peripheral blood and bone marrow as well as in maintaining the levels of the matrix-degrading enzyme matrix metalloproteinase 9 in the bone marrow vascular niche.15 In all of our IMPC cases, the cells lining the lacunar-like spaces surrounding the micropapillae strongly stained for CD31 but not for CD34, suggesting that they are newly formed immature blood vessels. The same cells were negative for D2-40, which is a sensitive and relatively specific marker for lymphatic endothelium in all parenchymatous organs.18

VEGF-A is a vascular-related protein widely regarded as a classic angiogenic cytokine because of its role in stimulating various endothelial cell responses necessary for angiogenesis. It belongs to the VEGF–platelet-derived growth factor gene family, which also includes VEGF-B, VEGF-C, VEGF-D, and VEGF-E. While VEGF-C and VEGF-D are involved in lymphangiogenesis,19 the expression of VEGF-A has been identified as an early change in the formation of new blood vessels.20 In our study, we used a monoclonal antibody that recognized the 189, 165, and 121 isoforms of VEGF. While the 2 smaller isoforms VEGF-165 and VEGF-121 are secreted proteins that act as diffusible agents, the larger isoform VEGF-189 remains cell associated. Presumably, VEGF and CD31 are related biomarkers in neoangiogenesis and may partially contribute to the biological aggressive behavior of IMPC. The coexpression of VEGF and CD31 in IMPC is a novel finding that warrants further investigation, particularly with respect to the possibility that targeted antiangiogenic therapy may be more efficacious in this subset of CRC.

In certain cases of IMPC, we observed collections of neutrophils infiltrating the micropapillae and occasionally spilling into the lacunar-like spaces, thus mimicking microabscesses. Neutrophils are a critical source of cytokines, such as interleukin 8, VEGF, and matrix metalloproteinases 2 and 9.21 Therefore, it is possible that they may also have a role in the neoangiogenesis associated with IMPC; however, further studies comparing IMPC with nonmicropapillary colorectal carcinomas are necessary to draw specific conclusions.

Conclusions

The results of this study demonstrate that the lacunar spaces surrounding tumor cells in colonic micropapillary carcinoma represent vascular channels and vascular endothelial growth factor A is produced by tumor cells. Our findings of strong CD31 and vascular endothelial growth factor A positivity in the lining tumor cells of the retraction clefts of invasive micropapillary colorectal carcinoma may represent neoangiogenesis around tumor clusters. This also suggests a prominent role of neoangiogenesis in the characteristics of early tumor progression, local invasion, and metastatic spread seen in invasive micropapillary colorectal carcinoma.

References


Molecularly targeted therapy may offer more tailored, personalized treatments for patients with breast cancer.

Advances in the Molecular Analysis of Breast Cancer: Pathway Toward Personalized Medicine

Marilin Rosa, MD

Background: Breast cancer is a heterogeneous disease that encompasses a wide range of clinical behaviors and histological and molecular variants. It is the most common type of cancer affecting women worldwide and is the second leading cause of cancer death.

Methods: A comprehensive literature search was performed to explore the advances in molecular medicine related to the diagnosis and treatment of breast cancer.

Results: During the last few decades, advances in molecular medicine have changed the landscape of cancer treatment as new molecular tests complement and, in many instances, exceed traditional methods for determining patient prognosis and response to treatment options. Personalized medicine is becoming the standard of care around the world. Developments in molecular profiling, genomic analysis, and the discovery of targeted drug therapies have significantly improved patient survival rates and quality of life.

Conclusions: This review highlights what pathologists need to know about current molecular tests for classification and prognostic/predictive assessment of breast carcinoma as well as their role as part of the medical team.

Introduction

For years the diagnosis and classification of breast cancer has been based on clinicopathological features such as tumor type and size, lymph node status, and histological grade. However, during the last few decades, significant advances have taken place in this area as we approach the era of personalized medicine.

Breast cancer comprises a heterogeneous group of tumors that significantly vary in their responses to treatment, presentation, and biology. For example, histologically similar tumors may have different clinical behavior and responses to treatment. Significant advances in personalized treatment during the last decade have been due to genomic analysis, which allows for the molecular study and classification of tumors and gives rise to the availability of biological targeted drugs such as trastuzumab for human epidermal growth factor receptor 2 (HER2)-positive breast cancer. However, the biological heterogeneity of tumors continues to be problematic because only a subset of patients with a particular type of tumor will benefit or respond to targeted treatments. In addition, most commercially available assays are designed to determine prognostic and predictive information in early-stage carcinoma and, thus, offer little clinical value to patients with advanced stage or aggressive variants of breast cancer. Therefore, it is important
to continue developing tests that can predict the risk of cancer recurrence as well as which patients will respond to specific therapeutic measures.

**Evaluation Using Traditional Methods**

Historically, breast cancer has been classified using histological grade. The first attempts to grade breast cancer based on histology were initiated by Greenough in 1925, followed by Patey and Scarff in 1928, Haagensen in 1933, and Bloom and Richardson in 1957. Elston's modified Bloom–Richardson grading system (also called the Nottingham system) is now used to grade breast cancer. However, interobserver variability and less-than-optimal reproducibility exist among pathologists regarding tumor grading. Nevertheless, tumor grading plays a role in the diagnosis and management of breast cancer because its value as an independent prognostic factor for overall survival rates in spite of tumor size and nodal status has been proven in numerous studies.

In addition to tumor grade, pathologists have traditionally classified breast carcinoma into histological types. The general consensus is that select types of carcinoma of the breast are associated with distinctive, biological characteristics related to clinical behavior. However, the classification and diagnostic criteria for each tumor type have also varied throughout the years. Select special-type carcinomas are uncommon; thus, they cannot always be included in long-term or molecular studies.

**Estrogen and Progesterone Receptors**

The estrogen receptor (ER) and progesterone receptor (PR) are prototypical tumor markers that have an immediate impact on systemic treatment decisions for patients with breast cancer. ER and PR are more commonly positive in low- to intermediate-grade tumors and in postmenopausal women. They are considered weak prognostic markers but strong predictive factors of tumor response to hormonal therapy (e.g., tamoxifen). PR is also an independent predictor of response because ER- and PR-positive patients have better responses to treatment than ER-positive, PR-negative patients.

ER and PR are measured using immunohistochemistry (IHC), which is the preferred method for measurement due to its wide availability and ease of use. However, it is estimated that nearly 20% of all ER and PR testing may not be accurate, and concerns regarding current testing, interlaboratory reproducibility, and possible false-negative or false-positive results exist. Several factors influence the accurate testing of ER and PR, including specimen type, fixation type and time, tissue decalcification, automated compared with manual procedure, antibody selection, threshold for positivity, and quality assurance and control. For optimal ER and PR testing, tissue must be sectioned at 5-mm intervals and fixed in a sufficient amount of 10% buffered formalin for at least 6 hours but no more than 72 hours. Cold ischemic time should be for no more than 1 hour. In addition, if nuclear staining is present in 1% or more tumor cells, then the results are positive. Every PR and ER IHC assay should include positive and negative controls.

**Human Epidermal Growth Factor Receptor 2**

The HER2 oncogenic protein is a transmembrane glycoprotein member of the HER family encoded by *ERBB2*. HER2 is expressed at low levels in several normal epithelia, including the breast. HER2 amplification and the accompanying protein overexpression occur in approximately 15% to 20% of breast cancers. HER2 overexpression, gene amplification, or both are independent prognostic markers of clinical outcomes. HER2 is also a predictive factor of tumor response to chemotherapeutic agents. Its status is typically evaluated to determine patient eligibility for anti-HER2 therapy. When used as monotherapy or in combination with other drugs or chemotherapeutic agents, HER2-targeted drugs have significantly improved survival and response rates.

HER2 status can be determined in formalin-fixed, paraffin-embedded (FFPE) tissue by assessing protein expression on the membrane of the tumor cells using IHC or by assessing the number of HER2 copies using in situ hybridization. For adequate testing, the fixation process should be initiated within 1 hour of tissue removal and the total fixation time should range from a minimum of 6 hours to a maximum of 72 hours. By IHC, scores of 0 and 1+ are considered to be negative, a score of 2+ is considered to be positive, and a score of 3+ constitutes a “gray zone” in which testing with an alternative methodology is necessary.

In situ hybridization methods include fluorescence in situ hybridization, chromogenic in situ hybridization, dual in situ hybridization, and silver-enhanced in situ hybridization. Some assays use a single probe to determine the number of HER2 copies; however, most assays also include a chromosome enumeration probe (CEP17) chromosome 17. Results are reported as follows:

- Not amplified; ratio < 2.0 with an average number of HER2 copies < 4.0 signals/cell
- Amplified ratio ≥ 2.0 or an average HER2 copy number ≥ 6.0 signals/cell
- Equivocal < 2.0 with an average HER2 copy number ≥ 4.0 and < 6.0 signals/cell

Several factors influence the accuracy of HER2 testing and tumor response to therapeutic agents. In the metastatic setting, response rates to trastuzumab are below 50%, and many patients initially responding to trastuzumab therapy go on to develop...
tumor recurrence and drug resistance.\textsuperscript{26-30} Although variable responses to trastuzumab may not all be related to inaccuracies in testing, the standardization of testing methodologies across laboratories is problematic; moreover, approximately 20% of HER2 testing may be inaccurate.\textsuperscript{26-30}

The most common factors that affect results include prolonged fixation in formalin, fixation in nonformalin fixatives, and tissue decalcification that may degrade DNA.\textsuperscript{21,25} Coamplification of CEP17 and HER2 can occur in breast cancer, causing miscalculation of the HER2:CEP17 ratio and, thus, underreporting of HER2 amplification.\textsuperscript{31} In addition, some tumors may demonstrate intratumoral heterogeneity for HER2 amplification. Although this definition has been challenged,\textsuperscript{19} HER2 tumor heterogeneity has been defined as a tumor that has at least 5% but no more than 50% of nonclustered tumor nuclei and a HER2:CEP17 ratio higher than 2.2.\textsuperscript{19,32} HER2 tumor heterogeneity may range between 5% and 15% of total cases tested and tumor heterogeneity may be more frequent (up to 27%) in breast carcinomas with an equivocal (2+) HER2 score.\textsuperscript{32,33}

Concordance between HER2 IHC (protein expression) and in situ hybridization (gene amplification) is required in at least 95% of cases.\textsuperscript{29} Among more than 1,500 patients whom they centrally screened, Perez et al\textsuperscript{14} found discordant results in approximately 4%. These findings may be due to amplification without overexpression, marked intratumoral heterogeneity, or protein overexpression without amplification. Response to anti-HER2 therapy in these cases is unknown.\textsuperscript{21,25,29,35}

**Human Epidermal Growth Factor Receptor 2 Testing Using Other Methodologies**

The HERmark (Monogram Biosciences, South San Francisco, California) assay uses a dual antibody approach to make quantitative measurements of HER2 content in FFPE tissue based on the VeraTag Technology platform (Monogram Biosciences). Compared with single antibody-based IHC methods, the dual antibody approach increases rates of specificity and sensitivity. HER2:HER2 homodimers and HER2 total protein are measured by the assay. Current testing methods identify patients eligible for trastuzumab.\textsuperscript{36,37} Alternatively, HERmark can distinguish subpopulations of patients likely to have different clinical outcomes; for example, those with higher levels of expression may have better outcomes than those with lower expression levels. Although the data is limited, studies suggested that compared with other marketed tests, HERmark can more accurately identify patients likely to respond to trastuzumab.\textsuperscript{36,37}

**Other Prognostic and Predictive Methods**

Many women diagnosed with early-stage, ER-positive, HER2-negative breast cancer receive systemic chemotherapy in addition to hormonal therapy following surgery to increase the likelihood of cure and reduce the risk of recurrence.\textsuperscript{38} However, it is possible that many of these women could be spared from the toxic adverse side effects of systemic chemotherapy if they were accurately stratified into groups that may or may not benefit from adjuvant chemotherapy.

Although it is not a molecular diagnostic test, Adjuvant! Online (www.adjuvantonline.com) is an Internet-based tool whose purpose is to help health care professionals and patients with early breast cancer review the benefits of adjuvant therapy following surgery, whether it is hormonal therapy, chemotherapy, or both. This online tool uses information such as estimates of comorbidity, tumor staging and characteristics (eg, ER status, size of tumor, number of positive axillary nodes), menopausal status, and age to provide baseline prognostic estimates of 10-year outcomes (with and without adjuvant systemic therapy).\textsuperscript{39} Most of the prognostic information is based on data from the Surveillance, Epidemiology, and End Results program, and projections of the efficacy rates of adjuvant therapy are based on data from the Early Breast Cancer Trialists’ Collaborative Group.\textsuperscript{38,40-42}

The Adjuvant! Online tool estimates the efficacy of endocrine therapy when used as monotherapy or in combination with systemic chemotherapy to determine likely outcomes.\textsuperscript{43} The tool can also model overall survival and disease-free survival rates as well as improvements seen in clinical trials. However, the Adjuvant! Online tool tends to overestimate the risk of recurrence in some patients.\textsuperscript{38,44}

The IHC prognostic model IHC4 uses the quantitative values of 4 standard IHC assays (ER, PR, HER2, and Ki67). The test was developed using data from a cohort of 1,125 ER-positive patients who did not receive adjuvant chemotherapy, had a genomic health Oncotype (Genomic Health, Redwood City, California) Recurrence Score, and had adequate tissue for the 4 IHC measurements (Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial).\textsuperscript{45} The test was further validated using data from another cohort of 786 ER-positive patients.\textsuperscript{45-47} To strengthen the IHC4 value, researchers combined it with clinicopathological parameters such as tumor grade, size, nodal burden, patient age, and type of endocrine treatment (aromatase inhibitors or tamoxifen), thus creating the IHC4 + clinical (IHC4+C) score.\textsuperscript{38,46} The IHC4+C score helps predict the residual risk of distant recurrence at 9 years in postmenopausal women with node-negative, hormone receptor–positive disease treated with 5 years of adjuvant endocrine therapy independent of the type of endocrine therapy;\textsuperscript{38} however, the test
cannot provide predictive information regarding drug of choice. Other researchers have concluded that the IHC4+C score provides additional prognostic information in this population that is at least as informative as Oncotype DX (Genomic Health). It provides a less costly and readily available methodology because 3 of the included IHC measurements are routinely performed in all patients diagnosed with breast cancer. However, variation in tissue handling, laboratory testing, and reporting of IHC tests hinders its generalized clinical use.

Barton et al compared the IHC4+C score with the Adjuvant! Online tool and found that the IHC4+C score additionally stratified patients according their the residual risk of distant recurrence among those already determined by Adjuvant! Online as being of intermediate risk. The IHC4+C score decreased the level of risk in more than 50% of individuals defined as having intermediate risk per the Adjuvant! Online to low risk, thus sparing some patients from adjuvant chemotherapy. In addition, Barton et al inferred that the IHC4+C score may have clinical utility in individuals labeled as high risk by Adjuvant! Online because the IHC4+C score downgraded almost one-half of these patients to the intermediate risk group and a select few were downgraded to the low-risk group. No additional usefulness of the IHC4+C score was seen in patients classified as low risk by the Adjuvant! Online tool, thus, this online tool may be sufficient for treatment decisions in these patients.

Gene-Expression Profiling

Gene-expression profiling has been used for more than 10 years to develop tests so that accurate and personalized clinical outcomes can be better predicted when compared with traditional pathological and clinical standards. In 2000, Perou et al described for the first time a molecular classification system for breast carcinomas, identifying 4 major molecular subtypes: ER-positive/luminal-like, basal-like, ERBB2-positive (HER2-enriched), and normal breast-like. Subsequent studies redefined the intrinsic molecular classification, resulting in a subdivision of the luminal type into types A and B. Luminal type A cancers have a low morphological grade and are predominantly positive for ER, whereas luminal type B cancers are also predominantly positive for ER, often have a high morphological grade, and may sometimes express low levels of hormone receptors. Amplification and high expression of ERBB2, along with several other genes of the ERBB2 amplicon, characterize HER2-enriched cancers. Most (but not all) basal-like tumors correspond to ER-, PR-, and HER2-negative tumors (Table 1). The normal breast-like subtype has not been reproducibly defined and is thought to be an artifact of having too few tumor cells and a background of normal breast tissue in the sample.

Although this classification gained acceptance, the initial testing methodology used messenger ribonucleic acid (mRNA) expression analysis in fresh frozen tissue, thus hampering its introduction into clinical practice. To identify subtypes using standard FFPE tissue specimens, other alternatives have been sought. Although intrinsic subtypes can be approximated using IHC stains, such as ER, PR, HER2, Ki67, epidermal growth factor receptor, and cytokeratins 5/6, 7, 8, 17, 18, and 19, the use of IHC for this purpose has not gained wide acceptance because the concordance with other molecular methods is not perfect and because of the complexity introduced when using multiple IHC markers on limited amounts of tissue.

Gene-expression profiling helps to identify genes with potential to be used as a molecular signature in guiding therapy and predicting patient prognosis. Popular breast cancer multigene predictor test platforms include IHC, fluorescence in situ hybridization, reverse transcriptase–polymerase chain reaction (RT-PCR),

| Table 1. Summary of Clinical and Pathological Features of Main Intrinsic and Molecular Breast Cancer Subtypes |
|---------------------------------------------------------------|-----------------|----------------|-----------------|-----------------|
| Intrinsic Type                                                | Luminal A       | Luminal B      | HER2 Enriched   | Basal Type      |
| Histological Grade                                           | Low to intermediate | Intermediate to high | High | High |
| Breast Carcinomas, %                                         | 40 | 20 | 20–30 | ~ 15 |
| Most Common Marker Results                                    | ER positive | PR positive | HER negative | Low Ki67 | ER (weaker) positive | PR positive or negative | HER2 positive or negative | Higher Ki67 | ER negative | PR negative | HER2 positive | ER negative | PR negative | HER2 negative | CK5/6 positive | EGFR positive |
| Prognosis                                                     | Good | Intermediate | Mutations in TP53 | High risk of relapse | Poor | Poor | High frequency of BRCA1 mutations |
| Targeted Treatment                                            | Hormonal therapy | Hormonal therapy | HER2-targeted therapies (eg, trastuzumab) | No targeted treatment options |
| Tumor Histology                                               | CK = cytokeratin, EGFR = epidermal growth factor receptor, HER2 = human epidermal growth factor receptor 2. |

214 Cancer Control April 2015, Vol. 22, No. 2
and microarray technology. Several tests are commercially available and many more are in development (Table 2). Some of the most clinically used methodologies will be discussed.

**Immunohistochemistry-Based Multigene Assay**

Mammosetrat (Clarient, Aliso Viejo, California) can help health care professionals make decisions regarding the use of chemotherapy as well as endocrine therapy in patients with ER-positive, early-stage breast cancer. Mammosetrat uses IHC to evaluate the gene expression of p53, SLC7A5, CEACAM5, NDRG1, and HIF9C, which have been selected from 700 gene targets in 3 individual cohorts, and has been comprehensively and clinically validated. The biomarkers are then analyzed using an algorithm that assesses risk for cancer recurrence, which is reported as low (7.6% likelihood of distant recurrence during a 10-year period), moderate (16.3% likelihood of distant recurrence during a 10-year period), or high (20.9% likelihood of distant recurrence during a 10-year period). This calculated risk of recurrence is independent of grade, lymph node status, and stage. In addition, the test appears to identify biological drivers of disease relapse that complement traditional pathological findings, such as lymph node status, tumor grade and size, and other biological markers (eg, HER2).

**Reverse Transcriptase–Polymerase Chain Reaction–Based Multigene Assays**

Oncotype DX is a prognostic, predictive, 21-gene profile, real-time RT-PCR assay performed by a central laboratory using FFPE samples of breast cancer. In individuals with ER-positive, HER2-negative, lymph node–negative carcinoma, the assay is used to determine their 10-year risk for disease recurrence, thus assigning them a recurrence score of low risk (< 18), intermediate risk (18–30), or high risk (≥ 31). Oncotype DX was developed after researchers identified and analyzed 250 candidate genes from 447 participants from 3 separate studies, eventually leading to its 21-gene profile that includes 5 reference genes as internal controls and 16 genes related to cancer. For the assay, the proliferation and ER pathways followed by the HER2 pathway play the most influential role in the calculation of the recurrence score.

Current guidelines advise oncologists to withhold chemotherapy in patients with low recurrence scores but to offer treatment to those with high recurrence scores. Patients with an intermediate recurrence score constitute a “gray zone.” To further clarify the risk of recurrence and benefit from chemotherapy in such patients, the Trial Assigning Individualized Options for Treatment (TAILORx trial) was initiated and is ongoing.

In addition to risk of recurrence, Oncotype DX also appears to predict chemotherapy benefits. In 2 large studies, lack of benefit from chemotherapy was associated with lower recurrence scores, and greater benefit from adjuvant therapy was associated with higher recurrence scores.

Oncotype DX is included in treatment guidelines from the American Society of Clinical Oncology and National Comprehensive Cancer Network. How-

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**Table 2. — Select Molecular Tests for Predicting Clinical Outcomes**

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>HERmark</th>
<th>Mammastrat</th>
<th>Oncotype DX</th>
<th>PAM50</th>
<th>MammaPrint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Assay</td>
<td></td>
<td>5-gene profile</td>
<td>21-gene recurrence score</td>
<td>50 classifier genes and 5 control genes</td>
<td>70-gene profile</td>
</tr>
<tr>
<td>Technology</td>
<td>2 distinct epitope-specific monoclonal antibodies Propprietary, proximity-based technology platform</td>
<td>Immunohistochemistry</td>
<td>Real-time RT-PCR</td>
<td>Quantitative RT-PCR</td>
<td>Microarrays</td>
</tr>
<tr>
<td>Laboratory</td>
<td>Centralized</td>
<td>Centralized</td>
<td>Centralized</td>
<td>Centralized</td>
<td>Centralized</td>
</tr>
<tr>
<td>Cost, $</td>
<td>~ 500–600</td>
<td>~ 4,000</td>
<td>Estimated to be 2,000–3,000</td>
<td>~ 4,000</td>
<td></td>
</tr>
<tr>
<td>Clinical Use</td>
<td>Prognostic Response to therapy</td>
<td>Prognostic</td>
<td>Prognostic Response to therapy</td>
<td>Prognostic</td>
<td>Prognostic</td>
</tr>
<tr>
<td>FDA Approval?</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

FDA = US Food and Drug Administration, FFPE = formalin-fixed, paraffin-embedded, RT-PCR = reverse transcriptase–polymerase chain reaction.
ever, the assay does have limitations. For example, it is validated in hormone receptor–positive breast cancer alone. It also has a high false-negative rate for HER2 that could lead to the underestimation of recurrence risk because such results influence the recurrence score. In some cases, the recurrence score does not correlate with histological features of the tumor (ie, low-grade tumors with high recurrence scores). In such cases, inflammatory cells or a cellular stroma may artificially increase the recurrence score in low-grade invasive breast cancers. In addition to these issues, the strength of this genomic assay has been challenged by some researchers who suggest that similar results and prognostic information can be obtained using less costly and more widely available testing methods (eg, IHC). However, because Oncotype DX is performed in a centralized setting, problems with test reproducibility and issues of interpretation are minimized.

In an effort to predict the risk of recurrence of ductal carcinoma in situ (DCIS), the Oncotype DX DCIS score was introduced. Approximately 30% of women with DCIS treated with breast-conservation surgery alone have local recurrence, and adjuvant radiotherapy has been shown to decrease the rate of recurrence by 50% in such individuals. The Oncotype DX DCIS score is a 12-gene profile (7 cancer-related and 5 reference genes) that estimates individual 10-year risk of local recurrence (DCIS or invasive carcinoma) to determine whether adjuvant radiotherapy is likely to be beneficial in women with DCIS treated by local excision with or without tamoxifen. The test has been validated for:

- Low- to intermediate-grade DCIS with tumor size ≤ 2.5 cm
- High-grade DCIS with tumor size ≤ 1.0 cm with a minimum negative margin width ≥ 3 mm or no tumor on re-excision

The risk is stratified into low (< 39), intermediate (39–54), or high (≥ 55) categories. When used in this context, the Oncotype DX DCIS can predict which women might be spared from adjuvant radiotherapy. However, the score does not account for other important clinical and pathological features that may influence risk of recurrence, including tumor size, margin width, tumor grade, and necrosis. Therefore, if Oncotype DX DCIS is ordered outside the clinically validated variables, its results may be inaccurate and potentially risky to patients.

The Prediction Analysis of Microarrays (PAM50; NanoString Technologies, Seattle, Washington) was introduced in 2009 by Parker et al. It uses a quantitative RT-PCR assay on FFPE tissue, measuring the gene expression of 50 classifier and 5 control genes to identify luminal type A, luminal type B, HER2-enriched, and basal-like breast cancer. In addition to classifying subtypes of breast cancer, the test yields a risk of recurrence score, taking into consideration the pathological tumor size and a subset of quantitative values for proliferation, luminal gene expression, ESR1 (ER), PGR (PR), and ERBB2.

The PAM50 breast cancer intrinsic classifier test is recommended for patients diagnosed with invasive breast cancer, regardless of their stage or ER status. PAM50 has been shown in multivariate analyses to be an independent predictor of survival rate in breast cancer, as well as independent and superior to ER status, tumor grade, lymph node status, and other variables.

However, PAM50 does not entirely correlate with IHC results because the test may classify some tumors that are HER2-positive by standard techniques, such as IHC or in situ hybridization, as luminal types A or B. Conversely, up to 30% of HER2-enriched tumors by PAM50 are clinically negative for HER2.

**Microarray-Based Multigene Assays**

The Symphony Genomic Breast Cancer Profile (Agendia, Irvine, California) is a comprehensive analysis of gene expression that includes MammaPrint (Agendia), BluePrint (Agendia), and TargetPrint (Agendia).

MammaPrint is approved by the US Food and Drug Administration to determine whether patients can safely avoid chemotherapy and its toxic adverse effects. BluePrint is a molecular subtyping profile that discriminates among 3 distinct molecular subtypes (luminal, basal-like, and HER2-enriched) and also guides treatment choices and combination therapies to optimize the treatment of breast cancer. TargetPrint tests for ER, PR, and HER2; therefore, it is used to determine whether patients with breast cancer are candidates for hormonal therapy or other targeted therapies.

For brevity, this article will focus its review on MammaPrint alone.

**MammaPrint**

MammaPrint is a microarray, in vitro, 70-gene expression profile that includes genes considered to be the hallmarks of local invasion, cancer-related biology, regulators of cell cycle, proliferation, metastasis, extravasation, survival in circulation, angiogenesis, and adaptation to the microenvironment. MammaPrint provides prognostic information, and its value is independent of conventional pathological and clinical factors (eg, HER2 status, tumor size, hormone receptor status). This test can also identify groups of patients at low risk within a node-positive population traditionally viewed as having a high risk for recurrence; thus, this assay has prognostic value in these individual patients. The test results are reported as low or high risk. Its prognostic use
is approved for women under the age of 61 who have ER-positive or ER-negative, lymph node-negative breast cancer, and whose tumors are smaller than 5 cm in size. Those considered to be low risk per the results of the test should be advised to avoid adjuvant chemotherapy and instead receive endocrine therapy alone, whereas those at high risk are generally advised to receive chemotherapy. The test is highly accurate for patients at low risk because the likelihood of progression to metastatic disease in these cases is low. However, for patients at high risk, the prediction of metastatic progression is not as precise because only approximately 25% of these patients will progress at 5 years, which may be partly due to the use of adjuvant therapies in these patients. In addition, the majority of ER-negative patients will be classified as being high risk based on the results of the assay.

To determine whether individuals classified to be at low risk by MammaPrint but at high risk via the Adjuvant! Online tool could safely avoid chemotherapy, the Microarray In Node negative and 1-3 positive lymph node Disease may Avoid Chemotherapy (MINDACT) and MammaPrint trial with Adjuvant Online! was designed to randomize individuals with breast cancer and discordant results to receive either hormonal therapy alone or hormonal therapy in combination with chemotherapy. At the time of writing, the results of this trial were not available.

The main limitation for the clinical use of MammaPrint is that it could not be performed on FFPE tissue, and tissue collected into an RNA preservative solution or fresh-frozen tumor samples was required. RNA retrieval from FFPE material is challenging due to the partial degradation of RNA during processing. Several investigators have tried to resolve this issue using archival FFPE tissue for clinical and research purposes, and their studies have demonstrated that applying DNA microarray analysis to FFPE tissue is possible, mainly due to improvements in technology and stringent protocols for tissue processing.

A study by Sapino et al validated the MammaPrint assay on FFPE tissue blocks. The authors compared the results between fresh tissue samples and FFPE tissue in an independent series of matched tissue from 5 hospitals and found an overall equivalence rate of 91.5%, a precision rate of 97.3%, a repeatability rate of 97.8%, and highly reproducible results between replicate samples of the same tumor and between 2 laboratories (concordance rate, 96%). These results may open the door for the wide clinical use of MammaPrint.

Role of Pathologists in the Era of Molecular and Personalized Medicine

Because specialized molecular tests are aimed to deliver personalized cancer care, pathologists are presented with new and unique opportunities to directly engage in research and patient care. The practice of pathology has shifted from being purely diagnostic to having a central role on the medical team. Traditionally, pathology departments have acted as “safe keepers” or “custodians” of tissue samples removed from patients for treatment reasons, diagnostic reasons, or both. New challenges regarding the ethical, regulatory, and legal aspects involved in tissue management require pathologists to be informed about the technical aspects of appropriate specimen management and suitability.

Molecular testing is performed for diagnostic, clinical (to obtain predictive or prognostic information), or research purposes. The ethical, legal, and technical issues involved in each of these applications are, to some extent, different. Molecular tests developed for clinical uses are subject to stringent regulations and can only be performed in laboratories certified by the Clinical Laboratory Improvement Amendments (CLIA). Typically, these tests do not require informed consent unless they are performed to detect heritable genetic mutations such as BRCA1/2. Tests developed for research purposes alone do not need to undergo the same degree of federal and state regulations and can be performed in smaller, noncertified laboratories. Tests performed in laboratories not certified by CLIA cannot be formally reported or used for clinical management decisions.

The success of molecular medicine depends on the quality of tissues to be tested. Preanalytic variables, such as tissue and biological sample collection, conservation, and transportation requirements, vary according to the type of test requested. Many molecular tests can be performed on FFPE tissue; few tests require fresh or frozen material. Fixation type, duration, and adequate tissue processing in the laboratory are crucial to ensure that genetic material is properly preserved. However, the role of the pathologist is not limited to supervision and guarantees of adequate tissue processing. Because of the broad spectrum of currently available molecular tests, no single laboratory is likely to be capable of offering them all. Pathology departments must have policies to establish how to handle “send-outs” for specialized tests, including clinical validity (medical necessity) of the test, adequate block and tissue selection, the release of tissue blocks or slides, covering the cost of supplies, shipping, and the test itself, among others.

The pathologist must also be aware of the amount of tissue needed for diagnostic purposes and make sure enough tissue remains to perform molecular studies, if needed, and when possible. The pathologist is also responsible for ensuring that enough tissue is available in the block to perform the requested test and to inform the testing facility about any deviation.
to specimen collection or processing (eg, prolonged fixation time). In addition, the pathologist should select the tissue block with the greatest amount of invasive tumor, highest tumor grade (unless otherwise specified by the oncologist), and the least amount of in situ carcinoma, stromal inflammation, and biopsy changes. Testing results should also be correlated with histological findings to identify and resolve any discordant results.87

The pathologist also has a duty to ensure that, when material collected for diagnostic purposes, treatment purposes, or both types of purposes is then used for research, enough tissue remains for clinical tests that may be needed or developed in the future and that such research has been reviewed and approved by the corresponding Institutional Review Board.

Conclusions

Developments in molecular medicine help to identify biologically different subtypes of breast cancer and characterize the risk of recurrence as well as to predict treatment responses among patients with breast cancer. In addition to traditional prognostic and predictive factors, such as estrogen and progesterone receptors, several assays that support breast cancer prognostication in clinical practice are available. These tests have been designed to help oncologists and patients make appropriate decisions regarding the use of adjuvant systemic therapy in addition to surgery. However, molecular tests should be used together and not as a substitute to well-established pathological and clinical variables used in routine practice to evaluate patient prognosis (eg, tumor grade, lymph node status). Technical advances in molecular medicine and the increasing number of molecularly tailored treatments have made it possible to potentially offer individualized treatment options to patients in the near future.

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**Introduction**

In the majority of cases, diagnoses of benign and malignant epithelial lesions of the breast are achievable using hematoxylin and eosin (H & E) microscopic sections alone. However, in some circumstances, such as in atypical and borderline breast lesions, as well as in core needle biopsy (CNB), this morphological distinction can be problematic. In addition, interobserver variability exists among pathologists when interpreting difficult and borderline lesions of the breast.\(^1,2\)

Therefore, using immunohistochemical (IHC) stains can be of help when dealing with these challenging lesions, particularly in cases for which the diagnosis carries a significant impact on management and prognosis. Pathologists involved in the diagnosis of breast cases should be familiar with diagnostic challenges they may encounter during their daily practice.

The purpose of this article is to describe the ancillary studies used for the diagnosis of benign, borderline, and malignant epithelial lesions of the breast as well as to describe studies used for the prognostic assessment of cancer. For the purpose of discussion, IHC stains used in breast pathology are divided into

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2 groups: those used for diagnostic purposes and those used to obtain prognostic/predictive information.

**Diagnostic Immunohistochemical Stains**

**Benign and Atypical Ductal Proliferative Lesions**

Diagnosing benign (usual and florid) ductal hyperplasia from atypical ductal hyperplasia (ADH) can be difficult on H & E examination. In addition to providing information regarding long-term cancer risk stratification, distinguishing the 2 groups in CNB specimens helps the clinical team plan the next step in the workup process.

The likelihood of finding a more severe lesion (ie, in situ, invasive carcinoma) on excision after the diagnosis of ADH ranges from 12% to 36%. Therefore, in most centers, the diagnosis of ADH is followed by excisional biopsy; consequently, making the correct diagnosis in borderline cases has great clinical implications. For several years, IHC has been used by pathologists for this diagnostic purpose. High-molecular-weight keratins (cytokeratins [CK] 5 and 6 and 34βE12) in conjunction with estrogen receptor (ER) IHC may be helpful in these borderline cases. Atypical proliferative lesions and in situ carcinoma lack staining with such keratins or they show occasional staining alone. By contrast, a strong and diffuse staining pattern indicates a benign process (Fig 1A).

When using these stains for the diagnosis of atypia in our practice, we have found that a potential pitfall is that cells with apocrine differentiation are generally negative. This finding is important when diagnosing atypia in columnar cell changes, which characteristically have apocrine features (Fig 1B). In this setting, the pathologist should rely on cytologic and architectural features on H & E examination alone. ER is sometimes used because, in the setting of benign breast tissue, ER shows scattered positivity alone in lobules and ducts. Conversely, in atypical and low-grade neoplastic processes such as ADH or low-grade ductal carcinoma in situ (DCIS), this stain tends to be diffusely and strongly positive (Fig 1C and 1D).

It is important to highlight that these IHC stains help classify a proliferative lesion into benign or atypical, and routine histological criteria must be followed to distinguish between ADH and DCIS.

**In Situ and Invasive Carcinoma**

The presence or absence of a myoepithelial cell layer around carcinoma cells is the basis for dividing tumors into in situ and invasive types. In most cases, distinguishing in situ from invasive carcinoma is straightforward. However, myoepithelial cell markers can be useful in diagnosing microinvasion, particularly in the setting of extensive high-grade DCIS or prominent inflammatory infiltrate associated with DCIS. In addition, certain entities are known to cause diagnostic challenges due to their complex architectural features. Invasive carcinoma or DCIS involving adenosis, invasive cribriform carcinoma or cribriform DCIS, and invasive carcinoma or solid papillary carcinoma are select examples (Fig 1E and 1F).

Any single myoepithelial cell marker is imperfect due to cross-reactivity with stromal myofibroblasts, vascular smooth muscle cells, or luminal epithelial cells. Therefore, at least 2 markers should be used in combination. The most appropriate combinations...
recommended in the literature have been a nuclear stain (p63) together with a cytoplasmic stain, such as smooth muscle myosin heavy chain, smooth muscle actin, or calponin.\textsuperscript{4,6,7} Among the cytoplasmic markers, smooth muscle myosin heavy chain appears to be more specific but is less sensitive than calponin. P63 has the advantage of being a clean and easy-to-read stain and it has good specificity rates in the setting of breast cancer.\textsuperscript{8} However, its staining pattern can be discontinuous, particularly around distended ducts with carcinoma in situ. In addition, most metaplastic carcinomas and, rarely, ductal carcinomas stain positively for p63.\textsuperscript{4} Despite this cross-reactivity, even when breast carcinomas stain positive for p63, staining tends to be patchy and of less intensity than adjacent myoepithelial cells.

Recent studies have also shown that qualitative differences may exist with regard to the myoepithelial cell markers expressed in different lesions; specifically, myoepithelial cells surrounding DCIS as well as benign sclerosing lesions have phenotypic alterations compared with myoepithelial cells surrounding normal ducts and lobules.\textsuperscript{9,10} P63, smooth muscle myosin heavy chain, and calponin all show reduced expression in these cases, potentially leading to the erroneous diagnosis of invasive carcinoma. In particular, the myoepithelial cells layer in small-sized biopsy specimens may not be present in the plane of the section.\textsuperscript{8} In addition, expansion of the ducts associated with DCIS could result in “attenuation” of the myoepithelial cell layer, and, in such a scenario, reactivity with myoepithelial markers may be absent.\textsuperscript{7} Paying careful attention to the type of lesion in question and using a panel that includes several myoepithelial cell markers could help avoid such potential diagnostic errors.

Myoepithelial cell markers have also been used to classify papillary lesions. Benign papillary lesions are characterized by a continuous layer of myoepithelial cells in the fibrovascular cores and at the periphery of the lesion. Although papillary carcinomas lack myoepithelial cells inside the lesion, the hallmark of in situ carcinomas is their presence at the periphery. Unusual entities, such as solid papillary carcinoma and encapsulated papillary carcinoma, can be devoid of “demonstrable” myoepithelial cells at the periphery. Therefore, discussion regarding the nature of these tumors has been taking place for some time, with some researchers noting that solid papillary carcinoma and encapsulated papillary carcinoma tend to behave as in situ carcinomas and, thus, are classified as such.\textsuperscript{11-13} Paying attention to morphological features on H & E and interpreting IHC stains in the correct context are key to making the correct diagnosis in such cases.

Microglandular adenosis is a benign lesion that defies the general rule of benign glands being surrounded by a layer of myoepithelial cells. Absence of a myoepithelial cell layer surrounding the glands, together with their tendency for haphazard growth into adipose tissue, makes this entity prone to being misdiagnosed as invasive carcinoma, specifically the tubular type.\textsuperscript{5}

Paying careful attention to subtle morphological features such as the commonly present, colloid-like secretions within the glandular lumina, as well as positivity of the epithelial cells for S100 can help in making the correct diagnosis. Of note, these lesions are negative for ER and progesterone receptor (PR), which would be unexpected in low-grade tubular carcinoma.\textsuperscript{6} This “unexpected” immunoprofile should raise questions in cases misdiagnosed as invasive carcinoma.

**Classification of Tumor Type**

**Ductal and Lobular Carcinoma**

Invasive and in situ carcinomas in the breast are divided into 2 main groups: ductal and lobular carcinomas. These 2 carcinoma types have major differences in anatomical distributions, long-term risk stratifications, and metastatic patterns. Consequently, to perform an appropriate preoperative workup, patient counseling, and treatment planning, an accurate diagnosis on CNB specimens is necessary.\textsuperscript{14-17}

IHC staining for E-cadherin, which is an adhesion molecule expressed in carcinomas of the ductal type, has long been used by pathologists. Ductal carcinomas show a strong membranous staining pattern, whereas lobular carcinomas are either negative or rarely display an aberrant staining pattern (Fig 1G and 1H). A small subset of lobular carcinomas (in particular, the pleomorphic type) can be positive for E-cadherin, and E-cadherin can show granular cytoplasmic staining in some lobular carcinomas. This is a potential pitfall in tumor classification because the finding is sometimes misinterpreted as positive staining. Careful attention to H & E findings as well as the location and the relative intensity of staining between tumor cells and benign ducts are all helpful in making the correct diagnosis.

The combination of E-cadherin and p120 (a catenin that binds to E-cadherin on the cell membrane and is essential for the formation of tight junctions) is superior to using E-cadherin alone when facing ambiguous cases.\textsuperscript{4} Ductal carcinomas will have a membranous pattern of staining, whereas lobular carcinomas show cytoplasmic staining. Because ductal and lobular carcinomas can be distinguished on H & E alone in the majority of cases or with the use of E-cadherin alone, we recommend using p120 for difficult-to-diagnose cases alone.
Special-Type Carcinomas and Spindle Cell Tumors

Typically, special-type carcinomas are diagnosed based on their morphological features on H & E examination. However, occasionally, IHC stains can be helpful in this setting, particularly for spindle cell tumors (Fig 2A–C). Recognizing metaplastic carcinoma without an obvious epithelial or heterologous element is difficult — and sometimes impossible — on H & E alone, and this is especially true for limited samples such as CNB. The differential diagnosis of spindle cell metaplastic carcinoma includes other spindle cell lesions, such as phyllodes tumor, fibromatosis, angiosarcoma, and spindle cell melanoma, among others. Keratin expression in metaplastic tumors can be heterogeneous and focal; therefore, a battery of CK stains (both pan-keratins and high-molecular-weight keratins), epithelial membrane antigen, p63, melanoma markers (eg, S100, melan A, HMB45), and additional markers to rule out other sarcomas (as needed) should be used in difficult cases.18-21

The identification of basal-like carcinomas is important in premenopausal young women because this type of tumor may be associated with hereditary breast and ovarian cancers and carries a poor prognosis.4 Most of basal-like carcinomas are triple negative (negative for ER, PR, and HER2), and variably express basal-type keratins (CK5/6, CK14, CK17), luminal-type keratin CK8/18, epidermal growth factor receptor, vimentin, and p53.18,19,22 This panel of immunostains can be used in clinical practice and represents a less costly, more readily available methodology in which to classify these tumors when compared with other commercially available assays.

Paget Disease of the Nipple

Most cases of Paget disease of the nipple are associated with underlying breast carcinoma (mainly DCIS); therefore, the diagnosis can be made on H & E. However, biopsy specimens in patients without a detectable underlying mass can be diagnostically challenging. The main differential diagnosis in such a rare setting includes melanoma and squamous cell carcinoma (Bowen disease).

CK7 immunostain is positive in almost all cases of Paget disease and is negative in squamous cell carcinoma. Typically, Paget cells express the same IHC characteristics of the underlying carcinoma.3 Because the underlying malignancy is frequently of a high-grade type, ER, PR negative, HER2-positive DCIS, HER2 immunostain is useful and positive in up to 90% of Paget cases. Therefore, the use of CK7 and HER2 is considered the most appropriate combination for confirming the diagnosis.23,24 Conversely, ER and PR immunostains are of no value in the differentiation of this entity from nonmammary lesions.

Select pitfalls must be kept in mind when evaluating CK7 immunostain. Both Toker and Merkel cells may be positive for CK7.25,26 Moreover, intraepidermal CK7-positive cells may represent an extension of benign lactiferous ducts into the nipple.4 Careful attention to the distribution and volume of positive cells may help distinguishing these benign entities from Paget disease. Melanoma can be ruled out by a panel of at least 2 immunostains, with the understanding that S100 is sometimes positive in Paget disease.27 Squamous cell carcinomas are negative for CK7 and positive for pan-keratin, high-molecular-weight keratins, and p63.
Metastatic Setting

Although breast cancer commonly metastasizes to other organs, metastases to the breast are rare and account for 0.5% to 2.0% of all breast malignancies.²⁸ In most cases, a history of malignancy is known. Melanoma is one of the most common tumors metastasizing to the breast, followed by carcinomas of lung and those of gynecological origin.²⁹ Absence of an in situ component as well as clinical history of another malignancy should raise suspicion for metastasis rather than primary breast carcinoma. The distinction is crucial because therapeutic management differs in these cases.

A commonly encountered situation is metastatic carcinoma of unknown origin to a nonbreast site. In these cases, metastatic breast carcinoma is usually in the differential diagnosis (Fig 2D). Breast carcinomas are likely to be CK7 positive and CK20 negative. However, a similar profile is seen in lung and gynecological tract carcinomas. Positivity for ER and PR can be helpful; however, these stains can also be positive in other tumors — in particular, carcinomas of a gynecological origin are positive for ER and PR (in which case, Wilms tumor 1 and paired box 8 can be helpful). When the differential diagnosis includes lung adenocarcinoma, thyroid transcription factor 1 (TTF1), and p63 (strong expression in sweat gland carcinoma, a panel of IHC that includes CK5, CK14, and mammaglobin, GCDFP15, and p63 is considered helpful). When the differential diagnosis includes lung carcinomas, can be used.³⁰

No single stain is unique to the breast. Gross cystic disease fluid protein 15 (GCDFP15) is relatively specific for the breast (also positive in salivary gland tumors and adnexal tumors of the skin), but it lacks sensitivity and shows a patchy staining, which becomes problematic in small-sized biopsy specimens. Mammaglobin is more sensitive than GCDFP15 but it lacks specificity. A combination of the 2 stains may be superior to either one alone; however, even when used in combination, up to 30% of tumors can be negative for both.³⁰ Of note, neither stain can be used to differentiate breast carcinoma from adnexal tumors of the skin (sweat gland carcinomas) or those of salivary gland duct origin. To distinguish between primary breast carcinoma and sweat gland carcinoma, a panel of IHC that includes mammaglobin, GCDFP15, p63 (strong expression in the latter group), and basal cytokeratins (CK5, CK14, and CK17) has been suggested as having highly sensitivity and specificity rates.³¹

GATA-binding protein 3 (GATA3) IHC is highly sensitive and fairly specific for breast carcinoma if urothelial carcinoma is not in the differential diagnosis. Rarely, staining for GATA3 has been reported in endometrioid adenocarcinoma.³²,³³ Of note, the rate of positive staining in breast carcinomas is highest in well-differentiated, ER-positive tumors, including lobular carcinomas (Fig 2E and 2F).

Prognostic/Predictive Immunohistochemical Stains

Hormone Receptors and HER2 Status

Hormone receptor and HER2 testing should be performed on all primary invasive breast carcinomas, as well as in recurrent or metastatic tumors. Multiple preanalytic and analytic factors can affect test results; however, this review only briefly summarizes the analytic component of these tests based on guidelines from the American Society of Clinical Oncology and the College of American Pathologists.³⁴,³⁵

All cases with at least 1% positive tumor cells for ER and PR have been associated with clinical response; therefore, they are classified as positive (Fig 2G).³¹ The College of American Pathologists requires providing quantitative information regarding the extent of positivity in all pathology reports.³¹ Quantification can be performed by simply providing the proportion of positive cells or using the Allred score or H score systems, both of which use the intensity and percentage of positive cells.³⁵

Staining patterns/intensities of HER2 immunostain have been categorized into 3 groups. Positive (3+) staining is defined by complete, intense, circumferential membrane staining (Fig 2H). Negative results include cases categorized as 1+ characterized as incomplete membrane staining that is faint/barely visible and in more than 10% of the invasive tumor cells, and 0 characterized by no staining or membranous staining that is incomplete and is faint/barely perceptible and within 10% or less of the invasive tumor cells.³⁵ Equivocal cases (2+) are those that display circumferential membrane staining that is incomplete, weak/moderate, or both and within less than 10% of the invasive tumor cells or complete and circumferential membrane staining that is intense and within 10% or less of invasive tumor cells.³⁵ If results are equivocal, then reflex testing should be performed using an alternate assay (ie, in situ hybridization if IHC was used as the initial test).³⁵

Conclusions

Immunohistochemical (IHC) stains provide information and aid in the differential diagnosis of challenging epithelial lesions of the breast. In addition, IHC can be used to obtain information relating to prognostic/predictive markers crucial for the treatment and prognostic assessment of patients with breast carcinoma.

IHC stains should be used selectively and judiciously and interpreted with the differential diagnoses and pitfalls in mind. Hematoxylin and eosin continues to be the most appropriate tool for diagnosing breast epithelial lesions.
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Reliable predictive assays will provide information to help inform clinical treatment decisions.

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Introduction
Cancer is the second leading cause of death in the United States, despite the many chemotherapy agents and hundreds of combinations available for treatment. However, health care professionals generally make treatment decisions based on standard protocols developed in clinical trials to successfully match a patient to a treatment regimen. This generalized approach can lead to a response, but oftentimes patients fail to show improvement. A predictive assay to help guide treatment decisions has the potential to lead to improved outcomes and fewer unnecessary adverse events endured by patients.

The treatment of cancer currently relies on classical chemotherapy regimens based on histology and targeted agents for patients who carry specific genomic alterations. In the era of genomics, we are learning that cancer is an individual disease and, with a push toward personalized medicine, treatment is shifting to a more tailored approach. However, genomic testing still fails to capture the factors that ultimately determine how tumor cells will behave inside the body.

One of these factors is intratumoral heterogeneity. Large-scale sequencing of solid cancers has revealed that different cells within a tumor can have distinct profiles, including in gene expression, proliferation, and metastatic potential, among others. These differences can contribute to treatment failures and have consequences related to personalized medicine. The impact on predictive assays lies in the sampling of tumors that commonly rely on biopsy specimens or small resections that do not fully represent the complexity of the tumor, thus resulting in the failure of drugs selected through personalized screening based

Special Technologies for Ex Vivo Analysis of Cancer

Jenny M. Kreahling, PhD, and Soner Altiok, MD, PhD

Background: Predictive assays for cancer treatment are not new technology, but they have failed to meet the criteria necessary for standardized use in clinical decision-making.

Methods: The authors summarize the use of predictive assays and the challenges and values associated with these assays in the clinical setting.

Results: Predictive assays commercially available in the clinical setting are not standardized, have significant obstacles to overcome, and cannot be relied upon by health care professionals due to the limited value these assays provide to the decision-making process for the treatment of patients.

Conclusions: A method that more closely recapitulates the human tumor microenvironment and accurately predicts response with high reproducibility would be beneficial to patient outcomes and quality of life.
on a small population of biologically identical cells.\textsuperscript{4,6} The information gained from studying tumor heterogeneity will be invaluable in optimizing predictive assays, such as serial biopsies, to gain a broader picture of individual tumor response to drug therapy.\textsuperscript{1}

Functional predictive assays providing information on personalized responses to drugs are needed to help guide treatment decisions and improve outcomes. Chemosensitivity assays offer potential in predicting treatment response; however, after more than 20 years, controversy still exists on the predictive value of such assays.\textsuperscript{7}

**Assay-Guided Therapy**

Chemosensitivity assays are described as any in vitro laboratory analysis that tests whether tumor growth has been inhibited by a known chemotherapy drug or a panel of drugs, including both classical and targeted agents and their combinations. All chemosensitivity assays share the same basic steps, including isolating tumor cells, incubating cells with drugs, assessing cell growth or survival, and interpreting the results.

These assays are often referred to as chemoresistance assays because ineffective drugs (resistant to treatment) are identified with significantly more accuracy more frequently than effective drugs (sensitive to treatment).\textsuperscript{8} This leads to questions regarding distinguishing drug-resistance assays from chemosensitivity assays and the reliability of these types of assays.\textsuperscript{2,5}

For any type of available chemopredictive assay, cancer tissue samples are obtained from biopsy specimens or during surgery. These samples are then dissociated, grown in a laboratory, and exposed to a range of concentrations of chemotherapeutic drugs, usually chosen by the ordering physician and regularly taken at least 3 weeks. The intent is that cancer cells treated in the laboratory will reflect the same response in the patient; however, these results are often ambiguous at best because the cancer cells show unexpected behavior, such as resistance to the drugs typically used to treat that particular cancer and sensitivity to drugs not generally used.\textsuperscript{10,11} These and other obstacles have slowed the use of chemopredictive assays in routine clinical testing.\textsuperscript{7}

**Cell Viability Assays**

Tissue culture–based chemopredictive assays have been used since chemotherapy was first used in cancer treatment. Growth inhibition or cell death has been used in previous iterations of assays of sensitivity to conventional chemotherapeutic agents.\textsuperscript{12} All assays use characteristics of cell physiology to distinguish between viable and nonviable cells to quantify cell death following exposure to a particular drug of interest. Drug doses used in the assays are variable, but all assays require drug exposures ranging from several fold below physiological relevance to several fold above physiological relevance. Some cell viability assays exist to examine chemosensitivity or chemoresistance; however, few are commercially available and periodically used in the clinic (Table).\textsuperscript{13}

The differential staining cytotoxicity assay involves mechanical disaggregation of cells from surgical or biopsy specimens.\textsuperscript{14} Three-dimensional fresh tumor cell clusters are cultured in anchorage-independent conditions and treated with the drugs of interest at 3 dose levels; the middle dose is that which could be achieved in therapy: 10-fold lower than the physiologically relevant dose and 10-fold higher from 4 to 6 days. At the conclusion of the culture period, fast green dye is added to the microwells, the contents are then sedimented onto permanent Cytospin (Thermo Fisher Scientific, Waltham, Massachusetts) centrifuge slides, and then they are counterstained with hematoxylin and eosin (H & E). The dead cells take up the fast green dye, and H & E allows the tumor cells to be differentiated from normal cells. Rates of drug sensitivity are measured by the ratio of live cells in the treated samples to the number of live cells in the untreated controls.\textsuperscript{8,13,14} This assay is capable of measuring both apoptotic- and nonapoptotic-mediated cell death in a population of cells; in addition, the assay can be applied to solid and hematological neoplasms, does not require a pure population, and can be used with a wide variety of drugs.\textsuperscript{15,16}

The ex vivo analysis of programmed cell death assay also measures apoptotic and nonapoptotic cell death markers in tumor samples exposed to chemotherapeutic agents. Tumor specimens obtained through biopsy specimens or surgical resections are

<table>
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<td>Drug sensitivity measured by the ratio of live cells in the treated samples to number of live cells in untreated controls</td>
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<td>End point of interest is cell death as assessed by number of cells differentially stained due to changes in the integrity of the cellular membrane</td>
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<td>3H-thymidine</td>
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<td>Histoculture drug resistance</td>
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<td>ATP bioluminescence</td>
<td>Measures ATP to quantify the viable cells in a culture using a luminometer. Decrease in ATP indicates drug sensitivity, whereas no loss of ATP suggests drug resistance</td>
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<tr>
<td>Chemosensitivity</td>
<td>Utilizing ATP quantification with a luminometer, cells are grown in a monolayer rather than a 3-dimensional matrix</td>
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ATP = adenosine triphosphate.
disaggregated using DNase and type 4 collagenase to yield tumor clusters of 50 to 100 cell spheroids. These microaggregates are thought to more closely approximate the human tumor microenvironment because they are not proliferated. Aggregates are then treated with dilutions of drugs and incubated for 3 days. After drug exposure is completed, a mixture of nigrosin B and fast green dye with glutaraldehyde-fixed avian erythrocytes is added to the cellular suspensions. 

The rationale for chemosensitivity assays is strongest where a variety of therapeutic options exists but no clear selection criteria have been chosen for any particular drug regimen in an individual patient. 

Some studies have reported a correlation between in vitro prediction or response and clinical response, and, although these studies may have internal validity, they cannot determine whether patients given assay-guided therapy or empirical therapy have different outcomes. To determine whether assay-guided treatment results in overall different outcomes than empirical treatment, it is important to take into account response rates, survival rates, the presence of...
adverse events, and patient quality of life. Chemosensitivity and chemoresistance assays are used in some centers for decisions related to future chemotherapy in situations with multiple equivalent treatment options available and when “the current level of evidence is not sufficient to supplant current standard of care chemotherapy.”27 A need exists to further validate these assays with direct evidence gathered from prospective trials comparing individuals empirically treated with those given assay-directed therapy. In this way, response rates, survival rates, the incidence of adverse events, and patient quality of life can all be taken into consideration.13,18,21,28-30

Concerns
Concern exists as to whether disaggregated tumor cells in culture represent the 3-dimensional structure of an intact tumor, including the tumor microenvironment, and their effect on drug treatment.2 Furthermore, cells exposed to a particular drug in a culture do not take into account the pharmacokinetics of drug metabolism and delivery in a human being. The reliability and consistency of chemopredictive testing are also concerning. Many studies have produced conflicting results, some of which have suggested a small benefit in assay-directed treatments, whereas others have demonstrated no difference at all.12 Specific problems with previous assays include the low percentage of successfully completed assays that yield clinically useful results, with rare interpretations that differed from standard of care, technical complexity that made their application beyond a single laboratory/institution unlikely, and the long time needed to study completion, thus delaying therapy. Reviews of the medical literature, government studies, and health care insurance evaluations concluded that the evidence is insufficient to support the use of chemopredictive assays in routine clinical practice and that such assays are not recommended, nor covered, by most insurance companies.16,21,28-30 Still, support continues for a predictive assay in the context of a clinical trial. Proposals for a successful assay include standardizing materials, testing a range of concentrations to provide a dose-response curve, applying simple techniques with the possibility of automation, and measuring cell survival using clear interpretation techniques.

Patient-Derived Xenograft Models
An advance over cell culture assay systems is the patient-derived mouse xenograft model that allows for the recapitulation of 3-dimensional tumor architecture and incorporates aspects of tumor stroma.12 Two major limitations of animal studies are their cost and intensity of labor required. Until recently, a comparison of xenograft responses with clinical outcome data had not yet been published.2,31 Previously, reports in soft-tissue sarcoma xenograft models showed low responses to conventional agents and were unable to find a strong correlation between selected genetic markers and chemotherapeutic effect.2 Furthermore, xenograft models of non–small-cell lung cancer were treated with 3 hemotherapeutic combinations.33 A 90% engraftment rate was achieved, and approximately one-half of the tumors were tested against all 3 chemotherapeutic regimens.33 The xenografts histologically resembled the primary tumor. Nearly one-third of the tumors were not sensitive to any of the tested combinations.33 In addition, a series of 11 patients received one of the tested combinations (vinorelbine and cisplatin), and 7 of the patients had recurrences, 6 of whom had corresponding nonresponsive xenografts.33 One xenograft was sensitive to the combination and also had a recurrence. Results from this assay took 6 to 8 weeks.33 The authors concluded that a correlation was present between the assay results and clinical data in the recurrence group and that xenograft-based testing may help identify new active combinations in non–small-cell lung cancer.33

In another study, 29 advance sarcoma xenografts were collected and 76% were engrafted.31 Of these 22, a total of 16 were correlated with patient outcomes, 13 of which had a positive correlation, including 6 tumors prospectively tested and 7 tumors tested after the study volunteer had already received chemotherapy.31 According to the authors, all 7 prospectively determined regimens, including 2 novel, nonstandard approaches, resulted in disease control or response in the xenograft system and were matched with clinical benefit to the 5 individuals who received these agents.31 Although this study had a small data set, its results are suggestive of a positive predictive value, and further investigation might identify tumor- and patient-specific treatments.2,31

Conclusions
The pursuit of reliable predictive assays is an important task, and the information these assays could provide would help inform clinical treatment decisions. However, after many years, these assays are still experimental and cannot be recommended for routine clinical use.7 Many still rely on traditional approaches of dissociating tumors and establishing cell lines maintained in vitro in serum-based growth media. Cultured cells are not completely representative of the parent tumor and such differences are of concern when predicting drug response as well as to the basic study of cancer. In particular, culture selection in cell lines may disturb the in vitro relationship between the cancer stem cell and its progeny, and it also removes tumor–stromal interactions essential to the 3-dimensional biology of solid tumors in vivo.12
The field is lacking studies published with randomized, prospective designs to evaluate the clinical utility of chemoresistance and chemosensitivity assays. The data are insufficient to determine whether use of a particular test to select chemotherapy regimens for individual patients will improve outcomes. Limitations exist and include sampling bias due to heterogeneity of tumors and insufficient biospecimen processing resulting in nonevaluable data; therefore, all chemosensitivity assays are currently considered to be investigational.13,18

The foremost priority in clinical oncology is providing personalized treatment approaches for more effective and better-tolerated therapies. One major step toward offering personalized therapy in cancer is the identification of actionable mutations so that health care professionals can select the most appropriate treatment for their patients. New technologies, such as next-generation sequencing (NGS), offer a comprehensive analysis of specific mutations present in the cancer of each individual patient and are likely to yield information regarding tumor biology and response to various treatment modalities. However, mutations identified through NGS analysis may not be actionable driver mutations, in that they do not lend themselves to specific and effective therapies.

Rather, we propose that combining ex vivo drug testing with NGS analysis may help distinguish random passenger mutations from true drivers. Fresh tumor samples can be directly treated ex vivo with a particular drug or drug combination for the targeted blockade of signaling pathways aberrantly activated through specific mutations in each cancer. The drug-mediated inhibition of proximal and distal signaling pathways accompanied by decreased cell viability would indicate a therapeutic opportunity with a specific drug for each individual patient. A similar approach could also be used as an enrichment strategy in designing clinical trials in which the tumor samples of study volunteers are tested ex vivo for their responses to drugs used in the clinical trials and then volunteers are assigned to clinical trials based on their ex vivo response to treatments. It is plausible that ex vivo functional assays may serve as the basis for inclusion or exclusion for clinical trials that can incorporate targeted agents in addition to cytotoxic therapy.12

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A review by Mahipal and colleagues of phase 1 trial enrollment among patients 65 years and older at the H. Lee Moffitt Cancer Center & Research Institute in Tampa, Florida, appears in this issue of Cancer Control. The study provides a recent and extensive — albeit not exclusive — exploration of the subject, encompassing 39 clinical trials (31 no-transplantation and 8 transplantation studies) conducted within a 10-year period involving 1,162 study participants, 380 of whom were between the ages of 65 and 91 years.

In their research, Mahipal and coauthors confirmed previous findings, including that:

- The mean age of individuals involved in phase 1 trials is approximately 10 years younger than the mean age of the general cancer population.
- Age between 65 and 75 years does not appear to affect the effectiveness or toxicity of drugs used in the no-transplantation setting.

In addition, they reported that the risk of toxicity increases with age in the transplantation setting. This finding is new but not unexpected because age is a recognized risk factor for toxicity and treatment-related mortality in patients receiving bone marrow transplantation.

The results of the review by Mahipal and colleagues are robust because they are based on the experience of 1 institution, which keeps carefully recorded data relating to outcomes and toxicities. The Cancer Control editors decided to publish this contribution as a reminder that chronological age — at least up to the age of 75 years — should not represent an impediment for patients to enroll in phase 1 clinical trials.

Like all retrospective analyses of data, the review of Mahipal and coauthors has several limitations, many of which the authors have acknowledged, including an inadequate assessment of age-related prognostic factors (eg, function, polymorbidity) and, more generally, an under-representation of the older population. In addition, the authors did not include study participants enrolled in cooperative phase 1/2 trials.

However, an additional limitation not mentioned was a failure to account for polypharmacy that, by itself, could represent a risk factor for chemotherapy-related toxicity. Popa et al reported that the risk of chemotherapy-related toxicity was increased when the risks of potential drug interactions were present, even when such an interaction was not associated with chemotherapy agents. Drug interactions can typically be expected in any patient who takes at least 8 medications every day. Another limitation is the absence of data related to phase 1 studies of targeted therapy, which is posed to be the most promising form of cancer treatment in the next decade. Yet another limitation is that the authors stopped their review at the year 2007 because they wanted to limit their analysis to studies involving cytotoxic chemotherapy. Some readers may question whether these old data are still relevant to the evolving landscape of cancer treatment.

Despite these limitations, the Cancer Control editors elected to publish this study for 3 reasons: (1) reaffirm the importance of physiological — rather than chronological — age for the enrollment of older individuals in clinical trials of cancer chemotherapy, (2) discuss which provisions would render more meaningful results from clinical trials with elderly participants, and (3) address the discussion as to whether a number of positions in phase 1 trials should be reserved for older individuals.

Undoubtedly, targeted therapy has produced significant improvement in the prognoses of numerous malignancies, but predicting the demise of cytotoxic chemotherapy would be premature because this treatment modality currently represents the only curative treatment for germ cell tumors, some forms of lymphoma and leukemia, and a number of breast, colorectal, and pulmonary cancers. It is reasonable to expect that cytotoxic agents will be the main bulwark against the spread of cancers with complex genomic alterations that may vary from cell to cell. Thus, phase 1 trials of cytotoxic chemotherapy agents are still relevant to individuals of all ages. When these trials are conducted in patients of advancing age, a wealth of desirable information may help contextualize the effects of a new drug.

All clinical cancer trials in the elderly, including phase 1 trials, should provide an estimate of physiological age (ie, life expectancy and tolerance of stress). This estimate may be obtained from a comprehensive geriatric assessment validated for this purpose. A reconciled list of medications may be important so that toxicities and unsuspected drug interactions can
be accounted for and documented. Although the comprehensive geriatric assessment currently provides the most reliable assessment of physiological age, some laboratory assays may help such determination in the near future. Among the most promising are the so-called inflammatory index and the leukocyte telomeres length.\textsuperscript{13-15}

One may ask whether second or third malignancies should exclude patients from enrolling in a phase 1 clinical trial. In approximately 20\% of patients with cancer 70 years of age or older, a second primary malignancy will be present.\textsuperscript{16} In the majority of cases, the second neoplasm may be indolent (eg, localized prostate cancer, low-grade lymphoma) and may not compromise patient survival for several years. Therefore, in my opinion, it is difficult to justify excluding these patients from enrolling in either phase 1 or 2 clinical trials. As the US population continues to age, one may consider a change in the recruitment criteria for clinical trials so that they more realistically represent the demographics of patients with cancer.

It is also desirable to examine the circumstances that may prevent older individuals from enrolling in phase 1 trials and to study how these barriers can be reversed.\textsuperscript{17} In addition to the increased prevalence of medical conditions that could potentially disqualify patients from trial participation, other barriers may include limited availability of transportation and health care professional and family prejudices.

Data are also needed related to the effects of phase 1 clinical trials in individuals 75 years of age or older. A single report of phase 1 studies collected data from 28 individuals aged 80 years and older, and found that, in this small patient population — the majority of whom was chemotherapy naïve — dose-limiting toxicities were higher than in younger individuals.\textsuperscript{18} Likewise, Schwandt et al\textsuperscript{19} reported that dose-limiting toxicities increased with age in patients older than 70 years but remained within an acceptable threshold. These limited findings should make us question whether a chronological age threshold exists beyond which we can expect a reduction in dose-limiting toxicities in the majority of patients. For more than 20 years, health care professionals have advocated that physiological age should supersede chronological age in the care of older patients and in clinical trials of cancer treatment.\textsuperscript{12} While I still stand by this principle, I recognize that the prevalence of the so-called oldest old is ever increasing,\textsuperscript{20} so it is legitimate to ask whether chronological age accurately reflects a critical reduction of physiological reserve in the majority of individuals older than 85 years of age.

Should we have phase 1 trials reserved for older participants? This question may sound more philosophical than practical. Personally, my position has been and continues to be that older individuals should not be excluded from phase 1 trials; however, at the same time, they should not receive special treatment. The goal of a phase 1 trial is to establish the maximum tolerated dose of a drug, and that is best accomplished without having to accommodate a special population such as the elderly. Thus, I would recommend that researchers reserve an adequate number of enrollment positions to individuals 70 years of age or older for phase 2 trials to establish how age may affect the pharmacology of a drug and whether a dose reduction may obtain similar results with less toxicity. One may argue that reserving a number of positions in phase 1 trials to older individuals, to those with a poor performance status, or those with significant comorbidity may produce a plethora of adverse events among the different populations and, thus, prevent the drug from misuse in clinical practice. I believe that the terms of the controversy should be exposed, but I doubt they can be solved in this context.

From time to time, the editors of Cancer Control publish studies with methodological flaws when the results of such studies are robust and important enough not to be ignored. I hope that the readership appreciates access to this information as well as the opportunity to discuss the important topics emerging from these studies.

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References
Effect of Age on Clinical Outcomes in Phase 1 Trial Participants

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Background: Most persons with cancer living in the United States are older than 65 years of age; however, in general, elderly persons are under-represented in clinical trials and outcomes data are lacking.

Methods: Outcomes data were analyzed of elderly participants (≥ 65 years of age) enrolled in phase 1 clinical trials and the results compared with those of younger patients. All consecutive, single-center, phase 1 oncology trials initiated and completed at the H. Lee Moffitt Cancer Center & Research Institute between 1997 and 2007 were included. Patient data (including survival, response, and toxicity rates) were extracted from a cancer registry database and electronic medical records at Moffitt Cancer Center.

Results: After excluding multi-institution trials, we analyzed 39 trials for a total of 1,162 enrolled study participants, 32.7% of whom were elderly. Among patients who underwent transplantation, median survival rates were worse in those who were elderly compared with those who were younger (44.9 vs 32.9 months; \( P = .0037 \)). However, in the no-transplantation setting, participants who were elderly had a median survival rate of 10.9 months (95% confidence interval [CI]: 8.9–13.1) compared with 8.8 months (95% CI: 7.9–10.3) in those who were younger (\( P = .15 \)). Both groups had similar overall response rates (15.2% vs 13.1%) and similar treatment-related mortality rates (1% vs 0.9%, respectively). Adverse events occurring among the elderly and younger participants were not statistically significant.

Conclusions: Survival, response, toxicity, and treatment-related mortality rates were not significantly different between the elderly and younger phase 1 trial participants in the no-transplantation setting. Regardless of the complex pharmacological profiles and logistical issues involved in treating the elderly population, our data imply that elderly study participants do at least as well as their younger counterparts, contributing to the justification of increasing the phase 1 trial enrollment of elderly patients.

Introduction

In the United States, the majority of all cases of cancer are diagnosed in people 65 years of age and older.\(^1,2\) Despite a growing elderly population, and a subsequently increasing cancer burden among the elderly, patients older than 65 years of age are frequently under-represented in oncology clinical trials.\(^3,4\) Limited data are available for this age group in terms of pharmacokinetics, toxicities, and the effectiveness of cancer treatments when compared with younger populations.\(^5,6\) In 1 trial of 16,396 patients, Hutchins et al\(^1\) reported that 25% of study participants were 65 years of age or older. Similarly, in another study of 28,766 persons with cancer who were enrolled in registration trials of novel cancer drugs or for new indications of cancer drugs already approved by the US Food and Drug Administration between 1995 and 2002, a total of 36% of patients were 65 years or older.\(^7\) Yet another study of 59,300 participants recruited in National Cancer Institute–sponsored, cooperative group trials between 1997 and 2000 reported that 32% of participants were 65 years of age or older.\(^8\)

Barriers to clinical trial participation among elderly persons include stringent exclusion criteria, logistical challenges, and misconceptions among health care professionals and patients about the risks of participation.\(^9\) Cancer in geriatric patients may be difficult to treat because of multiple factors, including age-related physiological changes, significant comorbidities, and possible drug–drug interactions. These complexities may sway a health care professional’s decision to approach elderly patients with cancer for participation.
in clinical trials. In addition, many physicians cite lack of information on elderly patients, calling on the need to increase data in patients aged 65 years and older. Although the under-representation of elderly persons enrolled in clinical trials is gaining attention, the actual involvement of elderly persons with cancer in clinical trials has yet to reach appropriate proportions. Recently, however, some randomized trials have specifically evaluated the role of therapy in elderly persons with cancer.

With regard to phase 1 trials, information on the enrollment of elderly participants and their clinical outcomes is limited. Phase 1 trials are crucial for evaluating the safety profile of a drug, determining a safe dosage range, and identifying any adverse events. For the majority of study participants, phase 1 clinical trials are safe and beneficial. In addition, prognostic indices are being developed to better predict survival rates and treatment tolerances in phase 1 studies. However, the impact of age in predicting response, treatment tolerance, survival rate, and possible complications remains largely unknown.

In this study, we sought to analyze the clinical outcomes of elderly participants (defined as age ≥ 65 years) enrolled in phase 1 clinical trials at the H. Lee Moffitt Cancer Center & Research Institute (Tampa, Florida) and compared their results with those of younger study participants (defined as age < 65 years).

**Methods**

All consecutive, single-center, phase 1 oncology trials initiated and completed at Moffitt Cancer Center between 1997 and 2007 were included in this analysis. The dataset was included until 2007 to primarily focus on chemotherapeutic trials. All trials were registered and received Institutional Review Board approval. All patients who gave informed consent to participate on a phase 1 study by December 31, 2007, were included on an intent-to-treat basis (ie, regardless of whether they received the study regimen). Patients who withdrew consent prior to receiving the study drug were not included in the cohort.

**Data Sources**

An OnCore system (Forte Research Systems, Madison, Wisconsin) database was used to generate the list of phase 1 trials conducted at Moffitt Cancer Center. Patients who consented to more than 1 clinical trial during the course of therapy were considered for the first Moffitt Cancer Center–only trial for which they received treatment. The Moffitt Cancer Center cancer registry database, case report forms (CRFs) containing original trial data, and electronic medical records (EMRs) were data sources used to extract individual patient data.

**Data Extraction**

Data were extracted using a standardized data extraction form. Variables of interest included demographic information, relevant dates for trial participation (enrollment, on treatment, off treatment, off study, and last contact), vital status, response, off-study reason, and toxicity grades and types. Random reviews of the dataset were performed to ensure accuracy of the data collection. More than 10% of the data was reviewed for accuracy.

Information on survival was primarily extracted from the cancer registry data. We supplemented and cross-referenced these data with the CRFs and EMRs. Data on response rate were extracted directly from the CRFs or EMRs for trials using the assessment noted by investigators. Investigator assessment was utilized for treatment decision-making on the trial and was considered accurate. For trials enrolling patients with solid tumors, investigators used the Response Evaluation Criteria in Solid Tumors for response assessment. Investigators used disease-specific response criteria for assessment in hematological malignancy trials. The CRFs for 1 myelodysplastic syndrome trial reported responses in terms of major and minor erythroid responses rather than complete and partial responses, among others. For consistency in reporting, we used the guidelines suggested by Cheson et al to convert these responses.

We extracted data on toxicities assessed by investigators according to the Common Toxicity Criteria (although the exact version varied depending on when the protocol was originally initiated) directly from CRFs or EMRs. Because toxicities were reported as adverse events (AEs) in CRFs, we extracted data for all grades 3, 4, and 5 AEs and noted whether they were attributed to treatment. Grade 3/4 toxicities were defined as being severe to life threatening and either definitely, probably, or possibly attributed to the study drug.

We used 2 methods to calculate mortality rate. Any death within 30 days following the administration of the last dose of the study drug was considered to be the 30-day mortality rate. The second method employed a direct extraction of the data from the CRFs or EMRs on death attributed to treatment. We reported both methods to account for patients who succumbed to disease within 30 days of being withdrawn from the study due to disease progression without having experienced any treatment toxicity.

**Statistical Analysis**

The outcomes of this study were overall survival (OS) rate, overall response rate (ORR), any grade 3/4 toxicity, and treatment-related mortality and 30-day mortality rates. For the purpose of this anal-
ysis, patients 65 years of age and older were considered to be elderly. Although analysis for benefits (ie, survival and response rates) was performed on an intent-to-treat basis, analysis for harms (ie, toxicities and treatment-related mortality rates) was performed per protocol. We planned a priori subgroup analyses on outcomes according to disease and treatment categories (eg, transplantation vs no-transplantation setting).

Survival was calculated using the Kaplan–Meier method from date of enrollment to last date of contact or death. A log-rank test was used to compare differences in survival between the subgroups. Differences in response rates and toxicity grades were calculated using the chi-square and Jonckheere–Terpstra tests, respectively. All reported P values were 2 sided, and a significance level of .05 was used. Statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, North Carolina).

Results

Study Selection

Between 1997 and 2007, a total of 147 phase 1 trials were opened at Moffitt Cancer Center. Of these, 108 were excluded due to multi-institution involvement, resulting in 39 trials (31 no-transplantation and 8 transplantation trials) for a total of 1,162 study participants at Moffitt Cancer Center alone (Fig 1). These study participants formed the intent-to-treat cohort. The median age in this cohort was 59 years (range: 18–91 years). Of the 1,162 study participants, 91% (n = 1,057) received treatment on study, 4.5% (n = 52) never received treatment, and 4.6% (n = 53) received an alternative treatment off study. Reasons for study participants not receiving treatment during the clinical trial included death, insurance issues, and the initiation of other therapies.

Baseline Characteristics

Table 1 lists the baseline characteristics of participants included in this study. Of the entire cohort (N = 1,162), 32.7% (n = 380) were elderly. The median ages of the elderly and younger participant co-

| Table 1. — Characteristics of the Volunteer Cohorts |
|---------------------------------|-----------------|-----------------|-----------------|
| Category                        | Age < 65 y (%)  | Age ≥ 65 y (%)  | P Value         |
| Overall                         | 782 (67.3)      | 380 (32.7)      | .0005           |
| Sex                             |                 |                 |                 |
| Male                            | 385 (49.2)      | 133 (35)        | < .0001         |
| Female                          | 397 (50.8)      | 247 (65)        |                 |
| Median age, y (range)           | 53 (18–64)      | 69 (65–91)      |                 |
| Hematological malignancy        | 303 (38.7)      | 107 (28.2)      | .0005           |
| Solid tumor                     | 479 (61.3)      | 273 (71.8)      |                 |
| Underwent transplantation       | 295 (37.7)      | 65 (17.1)       | < .0001         |
| No transplantation              | 487 (62.3)      | 315 (82.9)      |                 |
| Type of treatment               |                 |                 |                 |
| Chemotherapy alone              | 393 (50.3)      | 232 (61.0)      | < .0001         |
| Chemotherapy + transplantation  | 295 (37.73)     | 65 (17.1)       |                 |
| Immunotherapy                   | 14 (1.8)        | 17 (4.5)        |                 |
| Gene therapy                    | 59 (7.5)        | 47 (12.4)       |                 |
| Chemotherapy + radiation        | 21 (2.7)        | 19 (5)          |                 |
| Disease category                |                 |                 |                 |
| Hematological malignancies      | 303 (38.7)      | 107 (28.2)      | < .0001         |
| Lung cancer                     | 99 (12.7)       | 70 (18.4)       |                 |
| Melanoma and other skin cancer  | 83 (10.6)       | 43 (11.3)       |                 |
| Gastrointestinal cancer         | 74 (9.5)        | 73 (19.2)       |                 |
| Breast cancer                   | 63 (8.1)        | 7 (1.8)         |                 |
| Renal cancer                    | 31 (4.0)        | 21 (5.5)        |                 |
| Hepatocellular carcinoma        | 21 (2.7)        | 11 (2.9)        |                 |
| Soft-tissue sarcoma             | 17 (2.2)        | 5 (1.3)         |                 |
| Sarcoma                         | 16 (2.0)        | 3 (0.8)         |                 |
| Gynecological cancer            | 16 (2.0)        | 2 (0.5)         |                 |
| Mesothelioma                    | 10 (1.3)        | 19 (5)          |                 |
| Cancer of the endocrine glands  | 9 (1.1)         | 5 (1.3)         |                 |
| Other                           | 40 (5.1)        | 14 (3.7)        |                 |
Efficacy Outcomes

Response Rates: Among the study participants in the no-transplantation cohort, the ORR was 14%; 5.7% of study participants achieved complete response (CR) and 8.2% of achieved partial response (PR; Table 2). In addition, 30.4% of study participants achieved stable disease (SD). Response could not be evaluated in 15.4% of study participants. The primary reason for being unable to evaluate response included death or withdrawal from the study prior to restaging scans, screen failure, or the initiation of alternative therapies.

Among the elderly study participants in the no-transplantation cohort (n = 315), the ORR was 15.2% (CR: 7.3%; PR: 7.9%) and 33.7% had SD. The clinical benefit rate (CR + PR + SD) was 48.9%. Among younger study participants in the no-transplantation cohort (n = 487), the clinical benefit rate was 41.5%; CR was seen in 4.7%, PR in 8.4%, and SD in 28.3%. The differences in response rates were not statistically significant between the 2 age groups (P = .40).

Among the study participants who received stem cell transplantation, CR, PR, and SD were observed in 23.1%, 27.7%, and 23.1% of the elderly study participants, respectively. Progressive disease (PD) was observed in 1.5% of study participants, and 24.6% were not evaluable. Among the younger study participants, the ORR was 57.3% (CR: 31.5%; PR: 25.8%). SD was reported in 18.6% of these study participants and 7.5% had PD.

Overall Survival: In the no-transplantation group, the median OS rate was 10.9 months (95% confidence interval [CI]: 8.9–13.1) in the elderly cohort and 8.8 months (95% CI: 7.9–10.3) in the younger cohort (Fig 2; see Table 2). The difference in survival rates between the 2 age groups was not statistically significant (hazard ratio [HR] 0.89; 95% CI: 0.76–1.04; P = .145). No differences in the 2 age groups were seen on multivariable analysis. Tumor category (solid tumor vs hematological malignancy), sex, and type of treatment were significantly associated with outcomes in the multivariable analysis (Table 3).

In the transplantation group, elderly study participants had worse OS rates than younger study participants (HR 1.64; 95% CI: 1.17–2.29; P = .004). The median OS rate was 32.9 months in the elderly group and 44.9 months in the younger group. In the multivariable analysis, age and type of tumor were significantly associated with survival (see Table 3). When including both the transplantation and no-transplantation study cohorts, the OS rate was higher in younger study participants than in the elderly (median OS: 15.8 vs 13.4 months; P = .002).

Toxicity Outcomes

Among the elderly participants, 31% and 30% of them experienced grade 3 and 4 toxicities, respectively. Grade 3 and 4 AEs were observed in 31.7% and 34% of the younger study participants, respectively. No significant differences were seen in grade 3 and 4 AEs between the elderly and younger cohorts (P = .093). In the no-transplantation group, the incidences of grade 3 and 4 AEs among the elderly were 29.5% and 28.2%, respectively, and the incidences of grade 3 and 4 AEs among the younger patients were 34.2% and 31.9%, respectively.
and 4 AEs among the younger population were 30.6% and 27.3%, respectively. Table 4 lists the AEs of at least grade 3 experienced by all of the phase 1 clinical trial cohorts in the no-transplantation setting.

Gastrointestinal and metabolic nutritional toxicities were observed at higher rates in younger study participants, whereas general toxicities were higher in the elderly study participants. The toxicities were similar in the study participants undergoing transplantation; 78.6% of the elderly and 76.9% of the younger study participants experienced AEs of grade 3 or higher (P = .418).

### Treatment-Related Mortality

For those in the no-transplantation group, the 30-day mortality rate was 10% (30/300) among the elderly study participants and 9.2% (43/468) in the younger study participants, whereas treatment-related mortality rates were 1% (3/300) and 0.9% (4/468) for the elderly and younger cohorts, respectively. The differences between these rates were not statistically significant.

### Discussion

In our study cohorts (39 trials totaling 1,162 study participants), 32.7% of study participants were at least 65 years of age. Only 1 of the 39 studies evaluated was exclusively aimed at enrolling elderly participants. To align the discrepancy between the limited number of trials focused on elderly participation and the increasing number of elderly patients, more trials specifically designed for an elderly population are necessary.21 Due to the large percentage of patients with cancer aged 65 years and older,1 it is imperative to evaluate the safety and tolerability of possible treatments in elderly patients. This must be underscored by the fact that pharmacokinetics and pharmacodynamics significantly differ from those in young patients due to age-related biological changes.26 Furthermore, elderly patients frequently have more comorbidities and higher rates of medication use than their younger counterparts, thus making treatment options for cancer more complicated.

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Table 3. — Multivariable Analysis for Transplantation and No-Transplantation Cohorts

<table>
<thead>
<tr>
<th>Factor</th>
<th>HR</th>
<th>95% CI</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No transplantation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, ≥ 65 y vs &lt; 65 y</td>
<td>1.00</td>
<td>0.96–1.05</td>
<td>.8927</td>
</tr>
<tr>
<td>Male vs female</td>
<td>0.80</td>
<td>0.77–0.83</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Hematological malignancy vs solid tumor</td>
<td>0.39</td>
<td>0.37–0.43</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Type of treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotherapy + radiation vs chemotherapy</td>
<td>0.53</td>
<td>0.47–0.58</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Gene therapy vs chemotherapy</td>
<td>0.56</td>
<td>0.52–0.59</td>
<td></td>
</tr>
<tr>
<td>Immunotherapy vs chemotherapy</td>
<td>0.16</td>
<td>0.14–0.19</td>
<td></td>
</tr>
<tr>
<td><strong>Transplantation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, ≥ 65 y vs &lt; 65 y</td>
<td>1.90</td>
<td>1.73–2.09</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Male vs female</td>
<td>1.01</td>
<td>0.94–1.10</td>
<td>.7510</td>
</tr>
<tr>
<td>Hematological malignancy vs solid tumor</td>
<td>0.41</td>
<td>0.37–0.45</td>
<td>&lt; .0001</td>
</tr>
</tbody>
</table>

CI = confidence interval, HR = hazard ratio.
Several prognostic scores have been developed to predict clinical outcomes in patients participating in phase 1 clinical trials. The Royal Marsden Hospital prognostic score includes elevated lactate dehydrogenase levels, hypoalbuminemia, and more than 2 sites of metastasis as variables associated with poor prognosis. We performed a systematic analysis of all patients participating in phase 1 clinical trials at the MD Anderson Cancer Center (Houston, Texas). Gastrointestinal tumor type and an Eastern Cooperative Oncology Group performance status of 1 or more were both included as additional factors associated with prognosis. Study participants with none of these risk factors had a median OS rate of 24 months compared with 4.1 months in study participants with the highest risk scores. However, none of these scores included age as a prognostic factor.

To our knowledge, only 1 previous study has reported on survival rates in geriatric patients participating in phase 1 clinical trials. Zafar et al found that elderly patients who enrolled in clinical trials (n = 95) had a median OS rate of 8.4 months compared with elderly patients who consented but were ineligible for study inclusion (n = 114) whose median OS rate was 3.9 months. We performed a systematic analysis of all consecutive oncology phase 1 trials conducted at Moffitt Cancer Center between 1997 and 2007 and compared the benefits and harms in elderly and younger study participants. In the no-transplantation setting, we found no statistically significant differences in survival, response, toxicity, and treatment-related mortality rates between the age groups. To our knowledge, this is the first study to report toxicity data comparisons between geriatric and younger populations enrolled in phase 1 clinical trials. Moreover, we did not find any difference in the outcomes with regard to whether the age cutoff of 70 years was used instead of 65 years.

Among the patients undergoing stem cell transplantation, age has been demonstrated to be a major prognostic factor in several retrospective studies. Our results are consistent with prior studies, suggesting that elderly patients have significant worse survival rates than younger patients.

Our findings support the notion that age should not be used as a prognostic factor, particularly when determining eligibility for phase 1 clinical trials. Furthermore, our finding of a median survival rate of 10.9 months in elderly study participants not undergoing transplantation is comparable with results shown in previous studies reporting on the outcomes of participants enrolled in phase 1 clinical trials.

The current study also demonstrated that the incidence of high-grade toxicities is similar among elderly and younger study participants. By supporting the finding that elderly study participants do not have worse outcomes than their younger counterparts in phase 1 clinical trials, our study may provide the confidence health care professionals need to help their elderly patients decide whether to enroll in phase 1 clinical trials.

### Limitations

There are several limitations to our study, including its retrospective nature. All trials included were from Moffitt Cancer Center, thus providing a single-center perspective. It is unknown whether the data would differ if data from other centers were included. Other limitations include the high probability for selection bias among elderly patients. The low enrollment numbers of elderly patients, relative to their proportion of disease, may lead to enrollment of only the healthiest of elderly patients. This possible bias is impossible to accommodate for and difficult to assess.

Our study primarily included treatment with cytotoxic or immunotherapeutic agents rather than targeted agents, which are much more common today. However, the prognostic scores mentioned above that do not include age have been validated in patients...
receiving cytotoxic as well as targeted agents.

Although more studies are needed, our data demonstrate that clinical outcomes, effectiveness, and rates of toxicities are not substantially different between elderly and younger study participants in phase 1 clinical trials.

Conclusions

Participation in phase 1 clinical trials should not be hindered by age. Rather, efforts should be made to increase clinical study participation among the elderly patient population so as to decrease the gap between those who bear the disease burden and those enrolled in clinical trials. By accumulating evidence in the elderly population, health care professionals may be encouraged to treat their elderly patients in an appropriately aggressive manner. As the US population continues to age, information on treatment options for elderly persons has become — and will continue to be — increasingly crucial to patient care.

References


Primary Enteropathy-Associated T-Cell Lymphoma Type 2: An Emerging Entity?

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Summary: Intestinal T-cell lymphoma is a rare hematological malignancy that can present as primary intestinal lymphoma or as a manifestation of systemic disease. Primary involvement accounts for approximately 0.1% to 0.5% of all colorectal neoplasms. It is an aggressive disease with a poor prognosis and low survival rate. Inflammatory bowel disease, celiac disease, immunosuppression, and infectious etiologies, such as Epstein–Barr and human T-lymphotropic viruses, have been reported as risk factors, but no direct causal link has been established. Herein, we examine the case of a Hispanic man 69 years of age diagnosed with positive CD3, CD7, CD8, CD43, and Bcl-2 diffuse primary colorectal T-cell lymphoma. The patient did not exhibit a concomitant autoimmune or genetic disease. Because of the patient’s history of polyps, surveillance colonoscopy was performed and the diagnosis was confirmed.

Background
Gastrointestinal (GI) lymphoid tissues exist in the intestinal epithelium, lamina propria, submucosa, and lymph nodes. An accumulation of lymphocytic tumor cells can be seen in any of these tissue layers in GI lymphomas. Colorectal lymphomas manifest as either primary tumors or generalized lymphoma with associated colorectal involvement. Primary extranodal GI lymphomas are rare neoplastic processes accounting for 1% to 4% of all GI malignancies. Primary extranodal lymphomas of the GI tract are most common on the stomach (50%–60%) and small intestine (20%–30%). Cases of primary lymphomas of the colon are rare and most are of B-cell origin. Primary T-cell lymphomas of the lower GI tract are infrequent, representing approximately 0.1% to 0.5% of all colorectal neoplasms. Establishing a diagnosis of primary colorectal lymphoma requires histological confirmation that the lymphoproliferative neoplasm is isolated to the colon and the regional lymph nodes.

This hematological malignancy has a male predominance and a higher incidence in those 50 to 70 years of age. Inflammatory bowel disease, celiac disease, immunosuppressive states, and infectious etiologies, such as Epstein–Barr virus (EBV) and human T-lymphotropic virus (HTLV), have been reported to be risk factors; however, no direct causal link has been established.

Upon presentation, symptoms are typically nonspecific and may include changes in bowel habits, weight loss, abdominal pain, diarrhea, and rectal bleeding. Acute abdomen secondary to intestinal obstruction and tumor perforation requiring emergent surgical intervention have also been reported on initial presentation.

Early diagnosis is crucial in obtaining local control of the disease as well as to increase the likelihood of achieving remission with surgical intervention and adjuvant chemotherapy. Although colonoscopy is a crucial diagnostic tool in the diagnosis of colorectal disease, little is known of its value in the diagnosis of primary colorectal lymphoma.

Herein we present the case of a man diagnosed with diffuse primary colorectal T-cell lymphoma while being evaluated for unexplained weight loss.
sounds. No palpable organomegaly was present, nor were masses or lymphadenopathy. Findings on the rectal examination were also unremarkable. The patient had adequate sphincter tone with no evidence of fissures, nodules, or ulcers. Routine blood work was similarly unremarkable, with no evidence of anemia or leukocytosis, and the peripheral smear confirmed a normal leukocyte distribution.

In the setting of chronic dysphagia and weight loss accompanied by alcohol and tobacco use, upper endoscopy was obtained. Findings were remarkable for diffuse, severe nonerosive gastritis and mild nonerosive duodenitis without evidence of mucosal scalloping or other duodenal pathology. No evidence suggested the presence of esophageal lesions, rings, webs, or mucosal defects. Results from the rapid urease test (also known as the Campylobacter-like organism test) and gastric biopsies were negative for Helicobacter pylori.

Colonoscopy was also scheduled due to the patient’s remote history of colon polyps of unknown histology diagnosed more than 5 years prior to his current visit. Colonoscopy revealed the presence of a sessile polyp at the cecum (0.5 × 0.3 × 0.2 cm), 2 sessile polyps at the ascending colon (0.5 × 0.3 × 0.3 cm), 1 sessile polyp at the rectum (0.5 × 0.2 × 0.2 cm), and 1 polyp 15 cm from the anal verge (2 × 1 × 0.7 cm). The findings also demonstrated evidence of an erythematous and edematous fold 60 cm from the entry site and a flat lesion 27 cm from the anal verge (Fig 1).

All polyps were excised, and the histology was consistent with tubular adenomas. Histological examination of the colonic flat lesions biopsy samples revealed abundant intraepithelial lymphocytes (Figs 2 and 3), and immunophenotyping revealed an intraepithelial T-cell population positive for CD3 and negative for CD10 and CD5. Because of these findings, immunostains were performed to rule out lymphocytic colitis or neoplastic T-cell proliferation. The specimens were sent for further evaluation to the National Cancer Institute.

Histologic examination of the biopsies also identified infiltration by monomorphic, medium-sized lymphocytes with a rim of pale cytoplasm and round dark nuclei. Florid intraepithelial infiltration of the crypts was present as well as prominent intraepithelial lymphocytosis. Minimal inflammatory background without necrosis was also documented. Immunostains of the crypt infiltrate were highlighted by CD3, CD8, CD56, TIA-1, and T-cell receptor (TCR) γ; they were focally positive for perforin; and they had weak staining for CD2. CD20, CD4, CD5, TCR-β, granzyme B, and Epstein–Barr encoding region in situ hybridization were negative. Polymerase chain reaction studies showed evidence of clonal rearrangements of the TCR gene. The histology and immunophenotype supported the diagnosis of enteropathy-associated T-cell lymphoma (EATL) type 2 with evidence of the δ phenotype.

Staging computed tomography of the chest, abdomen, and pelvis showed no evidence of lymphadenopathy or extranodal organ involvement. Positron emission tomography was obtained and revealed no avid lesions. Results from HIV by enzyme-linked immunosorbent assay (ELISA) and antibodies against HTLV types 1 and 2 were negative. EBV serology was remarkable for negative antibodies against EBV viral-capsid antigen immunoglobulin (Ig) M, Epstein–Barr nuclear antigen, and early antigen with positive IgG antibodies against viral capsid, which has been associated with primary infection in the acute phase.

Positive serology against EBV brought contention regarding the diagnosis of EATL. However, no evidence suggested the presence of pleomorphic lymphoma cells, polymorphic inflammatory

Fig 1. — A large, flat ulcerated lesion with surrounding erythema and edema 27 cm from the anal verge.

Fig 2. — Section of colon showing neoplastic intraepithelial lymphocytes infiltrating the colonic crypts (hematoxylin and eosin, × 400).
infiltrate, angioinvasion, angiocentricity, or zonal necrosis — all of which are features of the nasal type of natural killer (NK)/T-cell lymphomas. Furthermore, the patient had TCR gene rearrangements positive for CD8, which are not seen in the nasal type of NK/T-cell lymphoma.

Celiac disease was excluded based on undetectable tissue transglutaminase antibody (< 1 U/mL) in the setting of an adequate IgA level (201 mg/dL; reference range, 131–407). Viral serology for hepatitis B and C (hepatitis B core antibody, hepatitis B surface antigen, hepatitis B surface antibody, and hepatitis C antibody by ELISA) was negative. Lactate dehydrogenase (LDH) levels were within normal limits. Bone marrow biopsy and aspirate were morphologically normal with no B or T cells. Monoclonal cells were apparent by flow cytometry.

Given the localized disease, the patient subsequently underwent total abdominal colectomy with ileorectal anastomosis (Fig 4). The pathology report identified 36 regional lymph nodes with focal areas of diffuse paracortical monotonous tumor consistent with a malignant lymphomatous tumor. Microscopic examination of the colon further demonstrated extensive lymphomatous involvement extending to the distal and radial margins.

The immediate postoperative period was complicated by multiple episodes of diarrhea that required aggressive hydration and antidiarrheal medications, including loperamide and diphenoxylate/atropine. Because of the extent of the disease, adjuvant chemotherapy was started with cyclophosphamide, hydroxydaunorubicin, vincristine, and prednisone (CHOP).

Response to the induction cycle was poor. Due to septic shock secondary to community-acquired pneumonia, the patient died 3 months after the diagnosis was established.

Discussion

Lymphomas are hematological malignancies with a wide variety of histological subtypes and a broad spectrum of clinical behaviors, aggressiveness, and prognosis. It is the eighth and ninth most common cause of cancer-related death in US women and men, respectively. The incidence rate for non-Hodgkin lymphoma (NHL) slightly increased among men between 2007 and 2011. In the United States, an estimated 80,900 new cases of lymphoma will be diagnosed in 2015; of those, 71,850 will be NHL. The GI tract is the main site for the extranodal dissemination of lymphoma and constitutes 40% of all cases. However, primary colorectal lymphoma is relatively rare, making up approximately 0.1% to 0.5% of all colorectal neoplasms and representing the third most common large bowel malignancy after adenocarcinoma and neuroendocrine tumor.

The most common location for colorectal lymphoma is the cecum and is suspected to be secondary to the abundance of lymphoid tissue in this anatomical region. Tumors of the descending and rectosigmoid colon account for approximately 25% of all colorectal lymphomas. EATL is the only well-defined clinicopathological entity of primary T-cell GI lymphomas. It represents a rare NHL of T-cell origin, and it accounts for less than 1% of all NHLs. The World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissue distinguishes between 2 types of EATL, with type 1 accounting for 80% to 90% of cases. The 2 subtypes, types 1 and 2, are classified based on histology, immunophenotype, and relationship to celiac disease. EATL is an infrequent type of aggressive lymphoma in most parts of the world, with an estimated annual incidence of 0.5 to 1 per 1 million people in Western countries. A higher incidence has been reported in northern Europe, where it overlaps...
with celiac disease. Type 1 is more common in Europe and type 2 is more common in Asia, whereas both EATL types are equally common in North America. EATL has a male predominance with a median onset during the sixth decade of life.

Type 1 accounts for two-thirds of all cases of EATL and is associated with celiac disease, which is a food intolerance disorder in Western populations that has a prevalence of 0.5% to 1%. The relationship between EATL type 1 and celiac disease is well established and is characterized by positive results on serology tests, HLA DQ2/8 expression in up to 90% of cases, and associated clinical findings such as dermatitis herpetiformis and hypoplasia.

Type 1
Microscopic findings of EATL type 1 are characterized by medium- to large-sized lymphocytes with round or angulated vesicular nuclei, prominent nucleoli, and moderate to abundant pale-staining cytoplasm. Neoplastic tissue tends to be infiltrated with abundant eosinophils and histiocytes, which may obscure the small numbers of tumor cells. Coagulative necrosis is common. The intestinal mucosa adjacent to the tumor frequently has enteropathic features consisting of villous atrophy, crypt hyperplasia, increased inflammatory cells in the lamina propria, and intraepithelial lymphocytosis.

Because EATL type 1 may present with monomorphic, small- to medium-sized cells, immunohistochemistry is generally helpful in distinguishing between the 2 subtypes. In general, type 1 tumors cells are positive for CD3, CD7, and CD103, but negative for CD5, CD4, and CD56; however, 10% of cases are positive for CD56. Tumor cells can also be positive or negative for CD8 and TCR-β, and a varying proportion of cells express CD30. Furthermore, EATL can conceal complex segmental chromosomal amplifications or deletions, and type 1 frequently displays deletions of 16q12.1 and gains of 1q, 5q, and 9q31.3.

Type 2
Type 2 accounts for 10% to 20% of all cases and has a broader geographical distribution. Typically, it occurs sporadically, but up to one-quarter of patients have a history of celiac disease and 30% to 40% express HLA DQ2/8. Histologically, type 2 is characterized by multiple foci of small, round uniform cells with dark nuclei and a rim of pale cytoplasm. Heavy infiltration of the intestinal crypt epithelium is present and a few admixed inflammatory cells may be seen; coagulative necrosis is absent. The adjacent intestinal mucosa shows villous atrophy and crypt hyperplasia with marked intraepithelial lymphocytosis involving both the crypt and surface epithelium. Immunohistochemistry of the tumor cells and intraepithelial lymphocytes on the adjacent mucosa will be negative for CD4 and positive for CD3, CD8, and CD56 in 80% to 90% of cases and TCR-β. This subtype is also characterized by chromosome 8q24 (MYC) amplifications and, less commonly, by gains of 1q and 5q.

Both types of EATL can be distinguished from reactive expansion by testing the clonality of the TCR genes.

Clinical Presentation
The clinical presentation of primary colorectal lymphomas is diverse and the duration of the presenting symptoms can widely vary. Patients with EATL most commonly present with abdominal pain, weight loss, diarrhea, vomiting, fatigue, and anorexia. In addition, a palpable abdominal mass, bowel perforation, bowel obstruction, B symptoms, and hemophagocytic syndrome have been reported in some cases. A history of adult-onset celiac disease with or without a disease-free period is typically present in patients with EATL type 1; childhood onset of celiac disease has also been associated with this subtype but not as frequently. In some patients, EATL is simultaneously diagnosed with celiac disease.

Our patient presented with unintentional weight loss alone, which has been reported in 27% to 80% cases in the literature. Serology tests for celiac disease were negative. Patients typically ignore this relatively nonspecific symptom for an extended period of time, thus further contributing to an advanced stage of disease at the time of diagnosis. In addition, performance status is variable at the time of presentation, with most patients having a WHO performance status of 1 to 3.

More than 90% of EATLs arise in the small intestine; the jejunum and proximal ileum are the most frequent sites. EATL of the large intestine, as in our patient, has been reported in approximately 16% to 18% of cases. Growth patterns tend to be diverse and lesions may range from multiple, ulcerating raised mucosal masses to 1 or more ulcers, large exophytic masses, strictures, or plaques. Classical lymphoma staging systems have failed to provide adequate prognostic treatment guidance for patients with EATL. Although they have not been validated, several case series have identified other novel adverse predictors of survival in patients with this type of lymphoma, such as elevated levels of C-reactive protein and LDH, tumor size larger than 5 cm, and a nonambulatory performance status.

Diagnosis
An exact diagnostic algorithm for EATL remains
undefined because of the lack of studies aimed at comparing the accuracy of the different diagnostic modalities. Colonoscopy is valuable in the diagnosis of primary intestinal lymphoma, but a high index of suspicion is needed for tissue biopsy and diagnosis. In 1 series, colonoscopy accurately diagnosed primary intestinal lymphoma in 23% of cases. Explanations for this may involve an insufficient specimen for pathological evaluation, because random biopsies in the absence of apparent pathology are often needed to accurately identify and determine the extent of lymphoma. Colorectal lymphoma is most often imaged using computed tomography, which can provide extraluminal and anatomical information regarding tumor size, depth of invasion, and regional lymph node involvement. Positron emission tomography may be of use for diagnosis and for follow-up patients, but its role has not been determined. A study published in 2012 showed that EATL type 2 is fluorodeoxyglucose (FDG) nonavid in 67% of cases, whereas type 1 is uniformly FDG avid. In this case, computed tomography and positron emission tomography imaging failed to discover any colorectal disease, thus highlighting the importance of high-quality colonoscopy in detecting rare GI pathologies that would have not been diagnosed otherwise.

Treatment

EATL has limited validated treatment strategies because of the lack of randomized clinical trials. Historically, treatment options have included surgical resections with or without anthracycline-based chemotherapy,10,13; however, results with these treatment modalities are limited. The National Comprehensive Cancer Network recommends an anthracycline-based combination therapy followed by autologous stem cell transplantation in eligible patients.19 A CHOP-like regimen, similar to what our patient received, has an overall response rate of 30% to 60%. With current treatment strategies, the median overall survival rate is approximately 10 months; the 5-year estimated survival rate is 20%. As in our patient, due to comorbidities, performance status, and his fragile state following total colectomy, only 50% of patients are eligible to undergo planned chemotherapy. Given data regarding their use in other CD30+ T-cell lymphomas, agents such as brentuximab vedotin, an anti-CD30 conjugated antibody, can be considered in patients with evidence of disease progression after induction therapy; a median duration of remission of 13.6 months has been reported. Khalaf et al reported on a patient with EATL successfully treated with brentuximab vedotin as salvage treatment who had a good response and disease remission at 9 months of follow-up. Due to its efficacy and tolerance, this medication could be considered as first-line treatment in CD30+ EATL in addition to current strategies to improve survival and response rates in patients whose tolerance to anthracycline-based therapy is expected to be poor. Additional case reports using alemtuzumab, a monoclonal antibody against CD52, and romidepsin, a histone deacetylase inhibitor, have shown promising clinical responses.

Conclusion

This case satisfies the histology and immunophenotypic criteria for enteropathy-associated T-cell lymphoma type 2 and highlights the difficulties in appropriately diagnosing T-cell malignancies. The incidence and localization rates of primary gastrointestinal lymphomas are known to vary based on race and geographical location, but their incidence and prevalence in the Hispanic population have not yet been determined. A significant increase can be seen in the incidence of EATL in the United States, most likely reflecting the increasing seroprevalence of celiac disease and better recognition of the rare types of T-cell lymphomas. It will be important to monitor the incidence of both EATL and premalignant conditions as evidence accumulates among whites and ethnic minorities. Although emerging data are encouraging, a significant need still exists for improved treatment modalities in this disease.

References


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Cost Effectiveness of Colorectal Cancer Screening Strategies

Shaan S. Patel and Meredith L. Kilgore, PhD

Background: Several screening tests are available to detect colorectal cancer (CRC) and reduce the incidence and mortality of CRC. The purpose of this review was to determine how current CRC screening strategies for CRC compare with no screening and whether agreement exists with regard to the cost effectiveness of different strategies.

Methods: Databases were searched for cost-effectiveness analyses focused on CRC screening strategies in the United States and published between May 2007 and February 2014. We analyzed the uses of fecal occult blood, fecal immunochemistry, and stool DNA tests, as well as sigmoidoscopy, colonoscopy, and virtual colonoscopy. A paired comparison of each screening strategy with no screening across each of the studies reviewed was conducted. A series of paired comparisons of the results reported in each of the studies is also included.

Results: When compared with no screening, all CRC screening strategies evaluated in this review were cost effective. There was disagreement as to which screening strategy was the most cost effective. However, sigmoidoscopy combined with fecal blood testing always dominated either strategy alone. Studies comparing colonoscopy with fecal blood testing, sigmoidoscopy, or both had mixed results.

Conclusions: Compared with no screening, all CRC screening strategies are more cost effective. Study results disagree as to which screening strategy should be the preferred method.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths among both men and women in the United States, and 132,700 estimated new cases of CRC and 49,700 CRC-related deaths will occur in the United States in 2015.1 The use of CRC screening tests can reduce the incidence and mortality of the disease through early detection so that the cancer can be removed before it progresses.2-5 Guidelines from the US Preventive Services Task Force (USPSTF), which were last updated in 2008 and are in the process of being revised, recommend screening for CRC using fecal occult blood testing, sigmoidoscopy, or colonoscopy starting at 50 years of age and ending at 75 years of age.6 According to these guidelines, evidence is insufficient to determine whether virtual colonoscopy, also called computed tomography (CT) colonography, or stool DNA testing is a cost-effective screening modality.6 Identifying the most cost-effective screening strategy for CRC can inform health care professionals as to which screening strategy to recommend for patients, and, thus, reduce the cost and maximize the effectiveness of CRC screening.

The purpose of this study was to conduct a systematic review, similar to the review of Lansdorp-Vogelaar et al,7 of the cost-effectiveness analyses of various CRC screening strategies. In contrast to the review of Lansorp-Vogelaar et al,7 this current review focuses on cost-effectiveness analyses conducted in the United States alone since May 2007. Our review examined the following questions:

1. How does the cost effectiveness of current CRC screening strategies compare with no screening?
2. Is there agreement as to the cost effectiveness of different screening strategies?

Materials and Methods

Identification of Cost-Effectiveness Analyses

The method for identifying studies and extracting data from the studies was described in previous studies conducting economic analyses of CRC screening tests.7-8 PubMed, the Cost-Effectiveness Analysis Registry, and the York Database of Abstracts of Reviews of Effects were searched for cost-effectiveness studies on CRC screening strategies, focusing on analyses published between May 2007 and February 2014. The literature search was limited to the last
We evaluated the following strategies for this question:

- No screening
- Annual fecal occult blood test (Hemoccult II and Hemoccult SENSA [Beckman Coulter])
- Annual fecal immunochemical test
- Sigmoidoscopy immunochemical test
- Combination sigmoidoscopy every 5 years and annual fecal occult blood test or annual fecal immunochemical test
- Colonoscopy every 10 years
- Colonoscopy with various time intervals and age of first colonoscopy
- Virtual colonoscopy every 5 or 10 years
- Virtual colonoscopy every 5 years with 2- or 3-dimensional imaging
- Virtual colonoscopy every 5 years with 100% or 50% adherence
- Stool DNA test every 2, 3, or 5 years

These screening tests were chosen due to their inclusion in the CRC screening guidelines from the USPSTF. Some studies specified whether sigmoidoscopy was followed with biopsy or not. Screening strategies from studies were eliminated if they were not distinct (eg, individualized colonoscopies). If a study did not specify the type of annual fecal occult blood test, then we assumed that the test was the annual Hemoccult II. If a study evaluated virtual colonoscopy, then cost and effectiveness estimates pertaining to extracolonic findings were eliminated. The commonly cited threshold of $50,000/LYG was set for assessing cost effectiveness.

**Assessing the Consistency of Study Findings**

We conducted a series of paired comparisons of the results reported in each of the studies included in this review. The differences in expected costs per person and the differences in life-years gained (LYGs) per person for each paired comparison were examined. The ICERs were examined for nondominated strategies using the following equation:

\[
\frac{\text{Total Effectiveness}_{\text{strategy A}} - \text{Total Effectiveness}_{\text{strategy B}}}{\text{Total Cost}_{\text{strategy A}} - \text{Total Cost}_{\text{strategy B}}}
\]

If strategy A was less costly and more effective than strategy B, then strategy A was characterized as dominant.

A threshold of $50,000/LYG was set for assessing cost effectiveness. The same strategies were similarly evaluated as in the first study question except for virtual colonoscopy and the stool DNA test. These 2 strategies were not included because the compar-

7 years to assess the cost effectiveness of the most up-to-date screening strategies used in the clinical setting. (The review by Lansorp-Vogelaar et al assessed studies up to January 2010.)

In PubMed, we searched for terms such as “colorectal screening” and “cost analysis,” and limited our search to English-language studies published May 2007 or later. This search identified 529 studies. The Cost-Effectiveness Analysis Registry was searched for the term “colorectal cancer screening,” for which 5 studies were identified with the same limitations as mentioned above. The York Database of Abstracts of Reviews of Effects was searched using the search terms “colorectal” and “cost,” for which 3 additional studies were identified using the same limitations. The reference list of the review by Lansorp-Vogelaar et al, which analyzed the cost effectiveness of CRC screening tests from 23 studies, was manually searched to identify studies not gathered by the database searches.

By evaluating information found in the titles, abstracts, and full texts of the studies gathered from the database searches, we excluded unoriginal studies, those that did not analyze at least 1 of the screening tests for CRC we were assessing, those that lacked an analysis of cost-effectiveness analysis, and those that analyzed special groups ineligible for routine screening. In contrast to previous reviews, we excluded studies that did not analyze a US population or report the cost in US dollars. We used these criteria for exclusion because the difference in price structures among health care systems in different countries makes a cross-comparison of the screening strategies uninformative. Collective agreement by the authors determined whether a study should be included if there was uncertainty.

**Comparing the Cost Effectiveness of Current Screening Strategies With No Screening**

A paired comparison of each screening strategy with no screening (eg, annual Hemoccult II [Beckman Coulter, Brea, California] vs no screening) across each of the studies was conducted to answer this question. The differences in expected costs per person and the differences in life-years gained (LYGs) per person for each paired comparison were examined. If one strategy was less costly and more effective than no screening, then it was characterized as being dominant. The incremental cost-effectiveness ratios (ICERs) were examined for nondominated strategies using the following equation:

\[
\frac{\text{Total Effectiveness}_{\text{strategy A}} - \text{Total Effectiveness}_{\text{strategy B}}}{\text{Total Cost}_{\text{strategy A}} - \text{Total Cost}_{\text{strategy B}}}
\]

If strategy A was less costly and more effective than strategy B, then strategy A was characterized as dominant.

A threshold of $50,000/LYG was set for assessing cost effectiveness. The same strategies were similarly evaluated as in the first study question except for virtual colonoscopy and the stool DNA test. These 2 strategies were not included because the compar-
isons were limited and their associated costs were higher than other screening strategies.

Results
Identification of Cost-Effectiveness Analyses
The search for relevant articles was initiated in February 2014; we were able to identify 543 studies. After analyzing the title of the studies, 267 of those studies were included in our analysis. After analyzing the abstracts of those studies, we chose 125 to include in our review, and their full texts were analyzed. Of these, 17 studies met the criteria for inclusion (Fig).

Each data element abstracted from each of the included studies can be found in Tables 1 and 2. Table 1 lists the perspective taken, models used, strategies evaluated, and basic results for each study. If a study used more than 1 model, then an additional entry was made in Table 1 for the given study. Table 2 provides further details, particularly on the sensitivity and specificity rates, costs for tests, and the types of sensitivity analyses performed. This review includes 13 studies not included in the review published in 2011 by Lansdorp-Vogelaar et al.

Cost Effectiveness of Current Screening Strategies vs No Screening
For 34% of comparisons made, CRC screening — regardless of the strategy evaluated — was less costly and more effective than no screening. The highest ICER, which was for stool DNA testing every 5 years, was $34,258 per life-year saved; nearly all other screening strategies had ICERs below $20,000/LYG compared with no screening. The complete results of this analysis are provided in the Supplemental Table (online).

Hemoccult II was less costly and more effective than no screening in 89% of the comparisons made. The annual Hemoccult SENSA and annual fecal immunochemical strategies either dominated no screening or had an ICER below $1,000/LYG. Overall, 60% and 78% of comparisons made showed that Hemoccult SENSA and an annual fecal immunochemical test, respectively, were less costly and more effective than no screening at all.

Sigmoidoscopy every 5 years either dominated no screening or had an ICER below $2,000/LYG. Overall, 64% of comparisons made showed that undergoing sigmoidoscopy every 5 years was less costly and more effective than no screening. Sigmoidoscopy every 5 years in combination with any annual fecal occult blood test (annual Hemoccult II or annual Hemoccult SENSA) either dominated no screening or had an ICER below $15,000/LYG. Approximately 50% of comparisons made showed that sigmoidoscopy every 5 years plus any annual fecal occult blood test was less costly and more effective than no screening. Approximately 44% of comparisons made also showed that sigmoidoscopy every 5 years plus an annual fecal immunochemical test was less costly and more effective than no screening. Sigmoidoscopy every 10 years had an ICER below $10,000/LYG.

Colonoscopy every 10 years beginning at 50 years of age either dominated no screening or had an ICER below $28,000/LYG. Overall, 18% of comparisons made showed that undergoing colonoscopy every 10 years was less costly and more effective than no screening. Virtual colonoscopy every 5 years did not dominate no screening in
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This study also included data from Sonnenberg et al.¹⁵
afFIT = annual fecal immunochemical test, aHemII = annual Hemoccult II, aHemSENSA = annual Hemoccult SENSA, COL10 = colonoscopy every 10 y, COL 10/40 = colonoscopy every 10 y starting at age 40 y, CRC-SPIN = colorectal cancer–simulated population model for incidence and natural history, CTC5 = computed tomography colonography every 5 y, CTC10 = computed tomography colonography every 10 y, CTC10/2D = 2-dimensional computed tomography colonography every 10 y, CTC10/3D = 3-dimensional computed tomography colonography every 10 y, CTC10/ER/without CAD = computed tomography colonography with a computer-aided design every 10 y (read by an experienced radiologist), CTC10/IR/without CAD = computed tomography colonography with a computer-aided design every 10 y (read by an inexperienced radiologist), DNA2 (SEPT9-2-well) = stool DNA test using 2-well methylated Septin 9 DNA assays every 2 y, DNA3 = stool DNA test every 3 y, DNA5 = stool DNA test every 5 y, MISCAN = microsimulation screening analysis, NS = no screening, SIG5 = sigmoidoscopy every 5 y, SIG10 = sigmoidoscopy every 10 y, SIGB5 = sigmoidoscopy with biopsy every 5 y, SimCRC = simulation model of colorectal cancer.
Table 2. — Model Inputs for Each Study Included
Table continued on next 3 pages

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any study, but it had an ICER below $22,000/LYG. Virtual colonoscopy every 10 years dominated no screening in 1 study,9 and the data from the rest of the studies reported an ICER below $23,000/LYG. Stool DNA testing every 3 years or 5 years did not dominate a no-screening strategy in any study, but these 2 strategies had an ICER below $35,000/LYG.

### Cost Effectiveness of Different Screening Strategies

When annual fecal immunochemical testing was compared with annual Hemoccult II testing, annual fecal immunochemical testing was either dominant or had an ICER below $50,000/LYG in 100% of the simulations from 5 different studies (Table 3). By contrast, annual Hemoccult SENSA testing dominated annual...
fecal immunochemical testing in 100% of the simulations from 2 different studies (see Table 3). The majority of comparisons made between sigmoidoscopy every 5 years and annual Hemoccult SENSA or fecal immunochemical testing showed that sigmoidoscopy every 5 years dominated in 100% of the simulations. When compared with annual Hemoccult II, sigmoidoscopy every 5 years had an ICER below $50,000/LYG in 70% of the simulations from 5 different studies (see Table 3).

### Table 2. — Model Inputs for Each Study Included (cont)

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<td>58,933.29</td>
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<tr>
<td>Distant, $</td>
<td>227,771.83</td>
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<td>85,310.10</td>
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<td>Measurement of effectiveness</td>
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<td>LGA</td>
<td>LGA</td>
<td>LGA</td>
<td>Life-y saved</td>
<td>Quality-adjusted LGA</td>
<td>LGA</td>
<td>LGA</td>
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<tr>
<td>Sensitivity analysis</td>
<td>1- and 2-way</td>
<td>1-way</td>
<td>Monte Carlo (10,000 simulation) and systematic</td>
<td>1- and multiway</td>
<td>Monte Carlo (10,000 simulation) and systematic analysis on all variables in the model</td>
<td>Monte Carlo (10,000 simulation)</td>
<td>Adherence rates 50% vs 100% for each test</td>
<td>Monte Carlo (10,000 simulation) and 1-way</td>
</tr>
</tbody>
</table>

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*This study also included data from Sonnenberg et al.¹⁵*

*CRC care depends on stage of cancer and phase of care.*

COL = colonoscopy, CRC = colorectal cancer, CTC = computed tomography colonography, FIT = fecal immunochemical test, HemII = Hemoccult II, HemSENSA = Hemoccult SENSA, LGA = life-y gained, NR = not recorded, SEPT9 = DNA SEPT9 analysis, SIG = sigmoidoscopy.
When sigmoidoscopy every 5 years plus any annual fecal occult blood test (Hemoccult II, Hemoccult SENSA, fecal immunochemical test) was compared with annual fecal occult blood testing alone or sigmoidoscopy alone every 5 years, sigmoidoscopy every 5 years plus any annual fecal occult blood test had an ICER below $50,000/LYG or dominated the other strategy in 99% of the simulations. The majority of simulations showed that sigmoidoscopy every 5 years plus annual Hemoccult SENSA testing either dominated or had an ICER below $50,000/LYG when compared with sigmoidoscopy every 5 years plus annual Hemoccult II testing. When comparing sigmoidoscopy every 5 years plus annual fecal immunochemical testing with sigmoidoscopy every 5 years plus either annual Hemoccult II or Hemoccult SENSA testing, the simulations showed that sigmoidoscopy plus annual fecal immunochemical testing had an ICER below $50,000/LYG in 27% of the simulations.

Colonoscopy every 10 years had an ICER below $50,000/LYG in 74% of the simulations when compared with any type of annual fecal blood testing. When compared with sigmoidoscopy every 5 years, colonoscopy every 10 years either dominated or had an ICER below $50,000/LYG in 87% of the simulations. When compared with sigmoidoscopy every 5 years plus annual Hemoccult II or Hemoccult SENSA testing, colonoscopy every 10 years dominated or had an ICER below $50,000/LYG in 53% of the simulations. Colonoscopy every 10 years was dominated or was not cost effective in 35% of the simulations when compared with sigmoidoscopy every 5 years plus annual fecal immunochemical testing.

**Discussion**

Using US data published in 17 studies since May 2007, this review reaffirms the results from previous reviews, such as the 2011 review by Lansdorp-Vogelaar et al,\(^7\) that any fecal occult blood test, sigmoidoscopy, colonoscopy, virtual colonoscopy, or stool DNA test was not cost effective in 35% of the simulations when compared with sigmoidoscopy every 5 years plus annual fecal immunochemical testing.

<table>
<thead>
<tr>
<th>Strategy</th>
<th>No. of Studies(^a)</th>
<th>No. of Simulations(^b)</th>
<th>No. Dominant(^c)</th>
<th>No. &lt; $50,000/LYG(^d)</th>
<th>No. &gt; $50,000/LYG(^d)</th>
<th>No. Dominated(^e)</th>
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<td>COL10 vs aHemII</td>
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<tr>
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<td>47</td>
<td>0</td>
<td>13</td>
<td>16</td>
<td>18</td>
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</tbody>
</table>

\(^a\)Number of studies evaluating a given comparison. \(^b\)Number of simulations in which model simulations were conducted for each comparison. \(^c\)Number of dominant = given a X vs Y comparison, the number of simulations resulting in X being dominant to Y. \(^d\)Number of studies with less than or more than $50,000/LYG = given a X vs Y comparison, the number of simulations resulting in X having an incremental cost effectiveness ratio less than or greater than $50,000/LYG when compared with Y. \(^e\)Three simulations showed COL10 to be less costly and less effective than SIG + aFOBT.

afIT = annual fecal immunochemical test, aFOBT = annual fecal occult blood test, aHemII = annual Hemoccult II, aHemSENSA = annual Hemoccult SENSA, COL10 = colonoscopy every 10 y, LYG = life-years gained, SIG = sigmoidoscopy, SIG5 = sigmoidoscopy every 5 y, SIGB5 = sigmoidoscopy with biopsy every 5 y.
is cost effective when compared with no screening. Evidence suggests any of the current screening strategies will reduce mortality when compared with no screening. For 34% of the comparisons made, CRC screening — regardless of the strategy evaluated — was less costly and more effective than no screening. The highest ICER calculated was approximately $35,000/LYG, and the corresponding screening strategy was stool DNA test (v 1.0) every 5 years. This strategy was still below the typical cost-effectiveness threshold.

In addition, the current review confirms that uncertainty still exists as to which screening strategy is optimal in terms of cost effectiveness. Potential reasons for the disagreement among studies as to which screening strategy is the most cost effective have been explained by previous reviews on the cost effectiveness of CRC screening strategies. Each study uses a separate model to simulate the natural history of CRC. However, Knudsen et al used 3 different models — microsimulation screening analysis, a simulation model of CRC, and a CRC-simulated population model for incidence and natural history — in their cost-effective analysis. The calculated ICERS between models were similar regardless of screening strategy; microsimulation screening analysis had a slightly higher ICER. In general, the decision analysis model used by Ladabaum et al reported results similar to other studies that used the Markov and Archimedes models. The use of different model inputs, such as dwell time of precancerous and cancerous phases, screening test costs, or screening adherence, may be potential factors for disagreement among studies. The sensitivity and specificity rates of screening tests also vary among studies, thus posing yet another potential reason for different results.

It is clear that sigmoidoscopy every 5 years plus annual fecal occult blood testing is cost effective when compared with either strategy alone. Colonoscopy every 10 years is cost effective when compared with annual fecal occult blood testing or sigmoidoscopy every 5 years. However, the results are mixed when colonoscopy every 10 years is compared with sigmoidoscopy every 5 years plus any annual fecal occult blood test.

Patients aged 50 years who have an average risk of CRC must be advised by their health care professional as to which screening strategy is cost effective. Based on this review, any screening strategy is more cost effective compared with no CRC screening, and high-quality evidence supports this recommendation. Furthermore, colonoscopy every 10 years or sigmoidoscopy every 5 years plus any annual fecal occult blood test should be the first 2 screening strategies recommended to patients, but evidence is of moderate quality to support this recommendation. Obviously, each screening strategy has its own advantages. Colonoscopy is more sensitive and allows access to the rectum and the entire colon, whereas sigmoidoscopy has fewer risks of complications and does not require sedation or extensive bowel preparation.

Limitations

Data extracted from each study came from simulations that assumed 100% adherence of using each screening strategy. Clinically, this does not reflect the actual adherence rate. An important gap in the literature is the need to incorporate compliance rates into cost-effectiveness estimations. It is likely that different screening strategies should be recommended to different patients, based on predicted adherence rates, or that health care professionals present their patients with different options for screening strategies.

Another limitation of this study is that we included studies of US patients alone and did not include international study populations. Due to the relative differences in prices between countries, the cost-effectiveness data from this review cannot be applied to other CRC screening strategies in other countries.

Conclusions

Solid agreement exists among all the studies reviewed that colorectal cancer screening is either dominant or cost effective compared with no screening at all, regardless of the screening strategy employed. We found considerable variation concerning which screening strategies would be preferred, subject to a prespecified level of cost per life-year gained deemed acceptable.

References


In this issue of Cancer Control, the Special Report by Lu and colleagues suggests that androgen deprivation therapy (ADT) may be associated with an increased incidence of colorectal cancer (CRC). We elected to publish this provocative paper for 2 reasons: (1) It supports the results of an analysis of Surveillance, Epidemiology, and End Results Program data with the weight of a Swedish cancer registry, which includes all cases of prostate cancer diagnosed since 1958 in Sweden, and (2) it is a reminder that some common and presumably innocuous interventions may have long-term complications. The association of CRC and ADT may, in fact, be the last nail in the coffin for the use of ADT in individuals with recurrences of prostate-specific antigen and without imaging evidence of metastatic disease. In these settings, the use of ADT does not improve survival rates, but its use has been associated with other serious complications, including the loss of libido, hot flashes, fatigue, sarcopenia, osteoporosis, diabetes, and an increased risk of infarction in the setting of coronary artery disease.2,3

A hypothesis suggested by Lu and colleagues is the possibility that estrogen therapy, which was widely used in Sweden prior to the introduction of gonadotropin-releasing hormone therapy, could reduce the risk of CRC. Undoubtedly, this article will elicit controversy, and Lu and colleagues have thus detailed the limitations and weaknesses of their Special Report.

The authors use 3 sources of information: (1) a Swedish cancer registry initiated in 1958 that includes nearly all cases of cancers diagnosed in Sweden, (2) a Swedish patient registry that began in 1964 for 2 counties and was then extended to the entire country in 1987 and includes surgical (but not medical) treatments, and (3) the Swedish total population registry that includes demographic data for the entire Swedish population since 1961. Prior to 1980, Swedish patients with prostate cancer were treated with estrogen therapy but with orchietomy, gonadotropin-releasing hormone therapy, or other treatments after 1980.4-7 Furthermore, the patient registry did not cover the entire Swedish population until 1987, so the authors infer that it is unclear whether patients underwent prostatectomy or orchietomy prior to that date. It is also unclear how many patients received radiotherapy in lieu of prostatectomy or how many experienced a recurrence following local treatment and underwent ADT as secondary treatment. Thus, the authors divided patients from the registries into 2 cohorts, ie, those diagnosed between 1960 and 1980 and those diagnosed between 1981 and 2008. Finally, no consideration was given to the fact that male life expectancy — and, hence, the possibility to diagnose new cases of CRC — has increased in Sweden since 1958.8

Despite these limitations, the incidence of CRC in Swedish men with prostate cancer has increased since 1980 and the results of this Special Report suggest that ADT is a likely culprit of this increase. Thus, we felt that these conclusions were important and robust enough to justify the publication of this Special Report by Lu and colleagues in the hopes that we can open a lively debate of the issue.

Is this study practice changing? Definitely not. It presents one more reason to avoid the use of ADT when it is not indicated, but it does not offer reasons to avoid ADT in conditions in which there are evidence-based benefits to this type of treatment. Such situations include using ADT as initial treatment for patients with metastatic prostate cancer in combination with radiotherapy in patients with locally advanced disease or in those at high risk of recurrence, as well as adjuvant treatment following prostatectomy in patients with involved pelvic lymph nodes.9

This Special Report is a reminder of the need to monitor long-term treatments throughout a patient’s lifetime, and this is particularly true of therapeutic options that may appear innocuous but may cause unwanted long-term consequences. Currently, vitamin D deficiency is considered to be the cause of a variety of conditions, including cancer, hematological disorders, and bone and cardiovascular diseases.8 As clinicians, we would be well advised to monitor the long-term effects of ongoing vitamin D supplementation. In yet another example of the importance of monitoring long-term treatments, we now know that vitamin E supplementation might have facilitated the development of prostate and lung cancers — the exact types of cancer it was purported to prevent!10 With the rising life expectancy of the global community (the global life expectancy has increased by 6 years since 1990), clinical epidemiology has become an invaluable instrument to detect the long-term effects of medical intervention.

This Special Report is also an opportunity to revisit a turning point in the management of prostate cancer.
Since the 1984 report of the Leuprolide Study Group suggesting that ADT in combination with leuprolide and a daily dose of 3 mg of diethylstilbestrol (DES), a synthetic estrogen, were comparable treatment options for the management of metastatic prostate cancer,10 estrogen therapy was all but banned from US practice. In our opinion, this approach might have been premature and ill advised. Estrogen therapy is associated with an increased risk of deep venous thrombosis (DVT), whereas leuprolide use increases the incidence of hot flashes10; however, at the time, the cost of estrogen was less than 1% of the cost of leuprolide. Furthermore, a study comparing leuprolide with a daily dose of 1 mg DES was never performed. Some evidence suggests that 1 mg and 3 mg DES may be equivalent but that the lower dose may be associated with a lower risk of DVT.11

Finally, estrogens do not cause many of the complications of gonadotropin-releasing hormone therapy, such as loss of libido, osteoporosis, and hot flashes.2,3 It may be too late to advocate a return to estrogen therapy, but the results from the Special Report by Lu and colleagues suggest that estrogen therapy might have had an additional advantage over ADT and that promoting the economic considerations of leuprolide might have trampled science and patient safety.

We must be skeptical about some of the study conclusions. For example, no biological or epidemiological evidence supports the claim that prostatectomy may have caused the incidence of CRC to increase. It is impossible to accept this conclusion without knowing how many patients received radiotherapy, hormonal therapy, or both following prostatectomy.

Some readers may question our decision to publish this Special Report despite its methodological flaws. As the editors of Cancer Control, we are privileged to place the importance of information over academic purity. Despite its flaws, we felt that the conclusion that ADT was associated with an increased risk of CRC was justified and robust, and, as such, the results of this Special Report are relevant to all health care professionals involved in the management of prostate cancer.

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References
Colorectal cancer (CRC) is the third most common cancer in the United States. The causality of CRC is multifactorial and includes genetic factors, lifestyle factors (diet, physical activity, smoking, alcohol consumption), obesity, metabolic syndrome, and sex hormones. In the study by Lu and colleagues published in this issue of *Cancer Control*, Swedish patients with prostate cancer were followed-up and an increased risk of CRC was observed in patients diagnosed with prostate cancer after 1980, which is when the use of androgen deprivation therapy (ADT; bilateral orchiectomy and gonadotropin-releasing hormone [GnRH] agonists) increased in Sweden. No increased risk of CRC was found in patients treated with estrogen therapy. Thus, Lu and colleagues concluded that ADT might be a causal factor for the increased risk of CRC seen in this cohort of patients. The similar but stronger and dose-dependent effect of ADT associated with an increased risk of CRC was also found by Gillessen et al.

Treatments for prostate cancer include active surveillance, radical prostatectomy, radiotherapy, or hormone therapy. ADT is a first-line treatment option for men with metastatic prostate cancer because it suppresses the binding of androgen to the androgen receptor. In Sweden, hormonal treatment for prostate cancer has changed over time; up until the 1980s, estrogen therapy was the predominant hormonal therapy prior to the introduction of ADT.

Animal studies have demonstrated that androgen may have a protective effect against colorectal carcinogenesis; by contrast, androgen deprivation may promote it. Evidence indicates that activating androgen receptors represses Wnt/β-catenin/T-cell factor signaling in colon cancer cells and is associated with a decreased risk of CRC. This process may influence competition among androgen receptors and T-cell factors for β-catenin binding; by contrast, administering antiandrogen therapy may instead reverse it. In fact, the expression levels of androgen receptors in CRC are lower than those found in samples of normal mucosa. Furthermore, testosterone deficiency has been linked to an increased risk of metabolic syndrome, diabetes, and cardiovascular disease in men; in addition, strong risk factors for CRC include obesity, hyperinsulinemia, type 2 diabetes, and metabolic syndrome. Research has also indicated that visceral adiposity accumulating during the short-term use of ADT is correlated with resistance to insulin (within 3 months for some cases) and increasing levels of circulating insulin.

Although clinical recommendations for CRC screening among patients with prostate cancer receiving ADT may be unnecessary at this time, health care professionals should be aware of the association between ADT and CRC in this patient population. It may be advisable for physicians to order relatively inexpensive laboratory studies (eg, fecal occult blood test) and suggest lifestyle modifications and early treatment options for lipid disorders in their patients with prostate cancer receiving ADT.

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References


Risk of Colorectal Cancer by Subsite in a Swedish Prostate Cancer Cohort

Yunxia Lu, MD, PhD, Rickard Ljung, MD, PhD, Anna Martling, MD, PhD, and Mats Lindblad, MD, PhD

Background: The relationship between sex hormone–related treatment for prostate cancer and the risk of colorectal cancer is controversial.

Methods: A prostate cancer cohort was initiated from the Swedish Cancer Registry of patients diagnosed between 1961 and 2008. Patients diagnosed with prostate cancer between 1961 and 1980 were generally treated with estrogen. The cohort diagnosed between 1981 and 2008 was further divided into 3 subcohorts of orchiectomy, prostatectomy, and other treatment. Standardized incidence ratios (SIRs) for developing colorectal adenocarcinoma were estimated and 95% confidence intervals (CIs) were used to compare relative risk among these patients and the general male population.

Results: Of 601,542 person-years of follow-up, 1,698 cases of colorectal adenocarcinoma were identified. Compared with the general male population, no association was detected in the cohort diagnosed between 1961 and 1980, whereas an increased risk of colorectal adenocarcinoma was observed among patients diagnosed with prostate cancer who received treatments other than estrogen. Following bilateral orchiectomy, the SIR was 1.30 (95% CI: 1.14–1.47); after prostatectomy, the SIR was 1.22 (95% CI: 1.04–1.43); among those who received treatment other than estrogen, the SIR was 1.37 (95% CI: 1.29–1.45). The increased risks were more apparent in cases of adenocarcinoma of the distal colon and rectum than in the proximal colon.

Conclusions: Patients with prostate cancer undergoing bilateral orchiectomy, prostatectomy, or other treatments, including antiandrogen therapy and radiation, may be at increased risk for colorectal adenocarcinoma.

Introduction

The role of the influence of sex hormones in the etiology of colorectal cancer (CRC) deserves attention, particularly regarding potential differences between the locations of such cancer. Based on separate embryological origins, and thus potentially divergent causal pathways, the colorectal intestine is often divided into the proximal colon (cecum, ascending colon, and transverse colon), distal colon (descending colon and sigmoid colon), and rectum. Accumulating evidence from epidemiological studies has demonstrated the differences in risk-factor profiles between men and women of cancers in the colorectal subsites with regard to dietary factors, obesity, and physical activity. The incidence of cancer of the proximal colon is 10% to 20% higher in women than in men at all ages, whereas men have a higher incidence of cancer in the distal colon and rectum. It is possible that sex hormones could differently influence cancers of the proximal colon, distal colon, and rectum. Epidemiological studies in women have shown that higher levels of the female sex hormones estrogen and progesterone are associated with a lower risk of developing CRC, particularly when exogenous steroid hormones are used, but few studies have associated sex hormones with risk of colorectal adenocarcinoma by subsite in men.

Previously, hormone therapy was a major treatment approach for prostate cancer, although hormone therapy does produce adverse events. Estrogen therapy and androgen deprivation therapy (ADT; including orchiectomy and gonadotropin-releasing hormone [GnRH] agonists) are 2 types of sex hormone therapies used in patients with prostate cancer. Theoretically, patients with prostate cancer treated with sex hormones may constitute an ideal natural human model for evaluating the association between external sex hormones and the risk of CRC. In 2010, a study found that patients with prostate cancer receiving ADT
had an increased risk of CRC compared with patients not receiving ADT.17 Due to confounding by indication, this association is still controversial. Further studies comparing patients with prostate cancer and the general population may provide more evidence.

Methods
Because of the nature of Swedish national health registries (see below for a detailed description of each registry used), we were able to identify all of the patients diagnosed with prostate cancer in Sweden between 1961 and 2008 and followed them up to the development of a second adenocarcinoma in the colon or rectum. The patients with prostate cancer were grouped into 4 treatment cohorts.

The aim of this study was to determine whether prostate cancer treatments, particularly sex hormone therapy, were associated with risk for CRC. The study was approved by the Regional Ethical Review Board in Stockholm, Sweden.

Data Sources
This study was based on data from 3 nationwide Swedish population-based registries, ie, the cancer, patient, and total population registries. The personal identity number, a unique 10-digit identification number assigned to each resident of Sweden, was used to link all of the patients between the registries.

Cancer Registry: The cancer registry was initiated in 1958 and includes the date of diagnosis, tumor site, and histological type of all malignant tumors diagnosed in Sweden. All newly diagnosed tumors in the country must be reported to the cancer registry by a clinician and a pathologist or cytologist. The completeness of the registry approaches 98% to 100%,18 and 99% of all tumors are morphologically verified.19 The cohorts included all men registered with a first diagnosis of prostate cancer. The years 1958 to 1960 were excluded to avoid inclusion of the prevalence of cancer cases when the registry began. All patients with any diagnosis of other previous cancer were excluded. Any subsequent cancer after prostate cancer was identified throughout the cancer registry, including the outcome of colorectal adenocarcinoma (proximal colon, distal colon, unspecified colon, and rectum).

Patient Registry: The patient registry was initiated in 1964 and covered 2 Swedish counties; starting in 1987, the registry covered 100% of Sweden.20 This registry has achieved accuracy and surgical completeness rates of 95% and 98%, respectively.21 Information on the surgical procedures following the diagnosis of prostate cancer was collected from this registry. The Swedish Classification of Operations and Major Procedures has been included in the patient registry since 1964, and it was used to identify patients undergoing bilateral orchietomy and prostatectomy.

Total Population Registry: The total population registry provides complete and continuously updated information on dates of emigration, immigration, births, and deaths since 1961. Data of censored cohort members who died or emigrated during follow-up were collected from this registry. However, these patients were censored from the date of first emigration or exact date of death.

Study Cohorts
The methods for treating prostate cancer changed in Sweden during the 1980s, with estrogen therapy being the predominant treatment strategy prior to 1980.22 Bilateral orchietomy was also an available standard treatment for prostate cancer between the years 1950 and 1980 in many countries, but orchietomy was not popular in Sweden during these years.23 Other treatments, such as ADT (orchietomy and GnRH analogues), radical prostatectomy, and radical radiotherapy, increased after the 1980s.24,25 The number of patients who did not receive treatment (active surveillance and watchful waiting) was low.27 Therefore, the cohort was divided into mutually exclusive subcohorts based on the definition of sex hormone treatments following prostate cancer diagnosis (Fig). The cohort diagnosed prior to 1981 included persons diagnosed with prostate cancer before 1981; a second cohort of patients included those diagnosed with prostate cancer between January 1, 1981, and December 31, 2008. The last subcohort was further divided into patients who underwent bilateral orchietomy, patients who underwent prostatectomy, and patients treated mainly by GnRH therapy, radiotherapy, or both assigned to the “other treatment” group.

Follow-Up
The follow-up of the cohort members started from the date prostate cancer was newly diagnosed or the start of specific treatments in the cohort, and patients were followed-up until any of the following end points: diagnosis of any cancer (except nonmelanoma skin cancer), emigration, age 85 years, death, or end of the study period, whichever came first. The end of the study period for the cohort before the 1980s was set to December 31, 1980, and to December 31, 2008, for all the other cohorts.22 The cutoff date (December 31, 1980) for the end of the first study period or the beginning of the second study period was arbitrarily selected based on the previously reported changes of treatment in Sweden.

Statistical Analyses
Person-years were calculated from the date of prostate cancer diagnosis (or specific treatment, eg, orchietomy) to the date of the study’s end point.
Standardized incidence ratios (SIRs) were estimated as a measure of relative risk when comparing the data from those diagnosed with prostate cancer and the corresponding general male population. The ratio was calculated based on the observed number divided by the expected number of newly diagnosed cases of CRC. The expected number of cases was calculated by multiplying the observed number of person-years by the age- and calendar year–specific incidence rates with 5-year intervals of the entire Swedish male population. SIRs and 95% confidence intervals (CIs) were calculated with the assumption that the number of cases followed a Poisson distribution. SIRs were separately estimated for cancers of the proximal colon, distal colon, unspecified colon, and rectum in each of the cohorts. We also performed sensitivity analyses with respect to the separate cohorts, which we assumed were exposed to various prostate cancer treatments. For the cohort diagnosed between 1961 and 1980, we analyzed the cohort members who were followed from 1961 to 1975, with the end point being 5 years earlier than the prior assumed cutoff date. For the 3 cohorts diagnosed after 1980, we performed analyses on patients who entered into the cohort from 1986 through 2008, with the start point being 5 years later than the prior assumed cutoff date. In the main analysis, we excluded the first year of follow-up after the diagnosis of prostate cancer because cancers diagnosed in the first year were particularly prone to detection bias and unlikely to be related to the treatment of prostate cancer.

All tests were 2-sided with a significance level of 0.05. Analyses were performed using the SAS statistical package, version 9.0 (SAS Institute, Cary, North Carolina).

Results

A total of 149,743 patients with prostate cancer were included in the final study cohort and information for the specific cohorts is shown in Table 1. The cohort diagnosed between 1961 and 1980 was composed of 33,373 patients with prostate cancer who were followed-up for 111,809 person-years until 1980, and the total cohort diagnosed with prostate cancer between 1981 and 2008 included 116,370 patients with prostate cancer and 489,733 person-years of follow-up since 1981. During follow-up, we identified a total of 1,698 cases of colorectal adenocarcinoma. Among them, 487 cases of adenocarcinoma were located in the proximal colon, 453 cases in the distal colon, 132 cases in an unspecified site of the colon, and 626 cases in the rectum. Age at entry and age at diagnosis of colorectal adenocarcinoma were significantly different among the groups (all \( P \) values < .01). Those in the prostatectomy cohort were younger than patients in the other cohorts.

**Estrogen Exposure**

Compared with the general population, the cohort diagnosed between 1961 and 1980 was not associated with the overall development of colorectal adenocarcinoma (SIR 0.98; 95% CI: 0.85–1.12) or when analyzed by subsite (Table 2). Results suggested a statistically nonsignificant decreased risk of adenocarcinoma of the proximal colon, but these results were mainly observed among patients with a short follow-up; however, a nonsignificant trend of increased risk with years of follow-up (\( P \) value for trend = .09) in the proximal colon was identified.

**Orchiectomy**

Between 1981 and 2008, a total of 21,917 individuals underwent bilateral orchiectomy and had an average follow-up period of 3.4 years (Tables 1 and 3). An overall increased risk of colorectal adenocarcinoma was detected in patients with 1 to 9 years of follow-up, but this risk decrease after 10 years of follow-up. A significantly increased risk
was observed for the overall rate of colorectal adenocarcinoma (SIR 1.30; 95% CI: 1.14–1.47) and for each subsite except for the proximal colon.

**Prostatectomy**

The prostatectomy cohort was composed of 16,521 patients who underwent prostatectomy between 1981 and 2008 and had an average follow-up period of 4.6 years (Tables 1 and 4). Similarly increased risks of colorectal adenocarcinoma overall and by specific subsites were observed among patients in the prostatectomy cohort.

### Table 1. — Basic Characteristics of the Prostate Cancer Treatment Groups

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of cohort members</td>
<td>Total person-y of follow-up</td>
</tr>
<tr>
<td></td>
<td>33,373</td>
<td>111,809</td>
</tr>
<tr>
<td>No. of colorectal adenocarcinoma cases</td>
<td>198</td>
<td>247</td>
</tr>
<tr>
<td>Average age at entry, y</td>
<td>71.9 ± 7.3</td>
<td>74.9 ± 6.4</td>
</tr>
<tr>
<td>Average follow-up, y</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>No. of colorectal adenocarcinoma cases at first year of follow-up</td>
<td>74</td>
<td>97</td>
</tr>
<tr>
<td>Average age at diagnosis of colorectal adenocarcinoma, y</td>
<td>76.2 ± 6.2</td>
<td>77.8 ± 4.7</td>
</tr>
</tbody>
</table>

Person-y incidence (95% confidence interval)

<table>
<thead>
<tr>
<th>Colorectal adenocarcinoma</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR  (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal colon adenocarcinoma</td>
<td>46</td>
<td>77</td>
<td>0.59 (0.46–0.73)</td>
</tr>
<tr>
<td>Distal colon adenocarcinoma</td>
<td>53</td>
<td>85</td>
<td>0.63 (0.49–0.78)</td>
</tr>
<tr>
<td>Rectal colon adenocarcinoma</td>
<td>59</td>
<td>124</td>
<td>0.47 (0.35–0.62)</td>
</tr>
<tr>
<td>Unspecified colon adenocarcinoma</td>
<td>20</td>
<td>43</td>
<td>0.46 (0.30–0.65)</td>
</tr>
</tbody>
</table>

Calculation based on the first year of follow-up had been excluded.

Incidence counted as per 100,000 person-y.

### Table 2. — SIRs and 95% CIs of Colorectal Adenocarcinoma in the Estrogen-Exposed Cohort Compared With the General Population (1961–1980)

<table>
<thead>
<tr>
<th>Colorectal Adenocarcinoma</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR  (95% CI)</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR  (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma of the Proximal Colon</td>
<td>177</td>
<td>152.4–201.8</td>
<td>1.16 (0.95–1.41)</td>
<td>329</td>
<td>288.1–370.2</td>
<td>1.16 (0.95–1.41)</td>
</tr>
<tr>
<td>Adenocarcinoma of the Distal Colon</td>
<td>46</td>
<td>33.1–58.1</td>
<td>1.39 (1.19–1.62)</td>
<td>77</td>
<td>57.4–97.2</td>
<td>1.39 (1.19–1.62)</td>
</tr>
<tr>
<td>Adenocarcinoma of the Rectum</td>
<td>53</td>
<td>39.3–66.2</td>
<td>1.38 (1.16–1.64)</td>
<td>85</td>
<td>64.4–106.2</td>
<td>1.38 (1.16–1.64)</td>
</tr>
</tbody>
</table>

Follow-up:

1–4 years: 129 | 0.96 (0.80–1.14) | 29 | 0.74 (0.50–1.06) | 40 | 1.04 (0.74–1.41) | 44 | 0.92 (0.67–1.24) | 16 | 1.67 (0.95–2.71) |

5–9 years: 59 | 1.10 (0.83–1.41) | 17 | 1.14 (0.66–1.82) | 16 | 1.12 (0.64–1.81) | 20 | 0.95 (0.58–1.46) | 6  | 1.76 (0.64–3.83) |

≥10 years: 10 | 0.74 (0.35–1.36) | 5  | 1.37 (0.44–3.19) | 3  | 0.88 (0.18–2.58) | 2  | 0.35 (0.04–1.27) | 0  | 0.00 (0.00–0.00) |

P value for trend: .99 | .09 | .98 | .34 | .49 |
Specifically, an increased SIR was found for the distal colon (1.44; 95% CI: 1.06–1.91) and rectum (1.36; 95% CI: 1.06–1.71).

**Other Treatments**

Among patients diagnosed with prostate cancer after 1980, a total of 77,932 did undergo bilateral orchiectomy or prostatectomy during the study period (see Table 1). Patients in the other treatment cohort had an overall increased risk of colorectal adenocarcinoma (SIR 1.37; 95% CI: 1.29–1.45) and for all subsites (Table 5). Specifically, an increased SIR of adenocarcinoma in the proximal colon was associated with age ($P$ value for trend < .01); a decreased risk was observed in association with years of follow-up ($P$ value for trend = .02; see Table 5).

**Sensitivity Analyses**

Analyses of the cohort diagnosed between 1961 and 1975 did not identify an association between estrogen treatment and overall or subsite risk of CRC, a finding consistent with the results from the originally defined cohort (diagnosed between 1961 and 1980). In the orchiectomy cohort, which included patients diagnosed between 1986 and 2008, increased SIRs of CRC were found, in line with the results shown in Table 3. In the prostatectomy and other treatment cohorts, similarly increased and stronger SIRs were observed when compared with the general male population.

**Discussion**

This nationwide Swedish cohort study identified an increased risk of colorectal adenocarcinoma for all anatomical locations among patients exposed to bilateral orchiectomy, ADT, radical prostatectomy, and radiotherapy. No clear association was detected among individuals exposed to estrogen.

Treatment for prostate cancer diagnosed after 1980 may be associated with an increased risk of colorectal adenocarcinoma, implying a possible connection to ADT, one of the most common treatments in Sweden used after 1980. Alternatively, a carcinogenic effect of radiotherapy could be related to an effect of surgical androgen deprivation, whereas the increased risk seen in patients not undergoing orchiectomy or prostatectomy could be due to medically induced androgen deprivation (ie, GnRH therapy). Higher SIRs were observed in the sensitivity analyses for the prostatectomy and other treatment cohorts, particularly in those with short-term follow-up. The results also indicated that other treatments such as GnRH therapy may play a role in the risk of CRC, although the probability value for such a trend did not reach statistical significance. Alternatively, a carcinogenic effect of radiotherapy for prostate cancer may be present; however, this represents exposure for which we have no data. Consistent results have also been found in a US study.

### Table 3. SIRs and 95% CIs of Colorectal Adenocarcinoma in the Prostate Cancer Cohort With Orchiectomy Compared With the General Population (1981–2008)

<table>
<thead>
<tr>
<th>Colorectal Adenocarcinoma</th>
<th>Adenocarcinoma of the Proximal Colon</th>
<th>Adenocarcinoma of the Distal Colon</th>
<th>Adenocarcinoma of the Rectum</th>
<th>Unspecified Colorectal Adenocarcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obs</td>
<td>Exp</td>
<td>SIR (95% CI)</td>
<td>Obs</td>
<td>Exp</td>
</tr>
<tr>
<td>247</td>
<td>190</td>
<td>1.30 (1.14–1.47)</td>
<td>58</td>
<td>55</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>0.88</td>
<td>1.06</td>
<td>1.16</td>
</tr>
</tbody>
</table>

**Age at diagnosis of prostate cancer, y**

| 41–64 | 26 | 17 | 1.55 (1.01–2.27) | 2 | 4 | 0.46 (0.06–1.66) | 10 | 4 | 2.48 (1.19–4.57) | 13 | 7 | 1.85 (0.98–3.16) |
| 65–74 | 132 | 97 | 1.36 (1.14–1.61) | 34 | 27 | 1.24 (0.86–1.74) | 32 | 23 | 1.40 (0.96–1.98) | 44 | 38 | 1.14 (0.83–1.53) |
| ≥75 | 89 | 76 | 1.17 (0.94–1.44) | 22 | 23 | 0.95 (0.59–1.43) | 22 | 17 | 1.26 (0.79–1.91) | 36 | 28 | 1.26 (0.89–1.75) |

**Follow-up**, y

| 1–4 | 158 | 127 | 1.24 (1.06–1.45) | 38 | 36 | 1.05 (0.74–1.44) | 37 | 29 | 1.26 (0.89–1.73) | 58 | 50 | 1.16 (0.88–1.50) |
| 5–9 | 75 | 49 | 1.53 (1.20–1.91) | 15 | 14 | 1.05 (0.79–1.72) | 24 | 11 | 2.10 (1.34–3.12) | 30 | 19 | 1.59 (1.07–2.27) |
| ≥10 | 14 | 14 | 1.00 (0.55–1.68) | 5 | 4 | 1.17 (0.38–2.73) | 3 | 3 | 0.88 (0.18–2.57) | 5 | 5 | 0.96 (0.31–2.24) |

**P-value for trend**

| .15 | .99 | .14 | .51 | .49 |

*First year of follow-up was excluded.

CI = confidence interval, Exp = expected, Obs = observed, SIRS = standardized incidence ratio.
Table 4. — SIRs and 95% CIs of Colorectal Adenocarcinoma in the Prostatectomy Cohort Compared With the General Population (1981–2008)

<table>
<thead>
<tr>
<th>Age at diagnosis of prostate cancer, y</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>41–64</td>
<td>156</td>
<td>127</td>
<td>1.22 (1.04–1.43)</td>
<td>30</td>
<td>35</td>
<td>0.86 (0.58–1.23)</td>
<td>48</td>
<td>35</td>
<td>1.44 (1.06–1.91)</td>
<td>71</td>
<td>52</td>
<td>1.36 (1.06–1.71)</td>
</tr>
<tr>
<td>65–74</td>
<td>73</td>
<td>63</td>
<td>1.16 (0.91–1.46)</td>
<td>13</td>
<td>16</td>
<td>0.79 (0.42–1.36)</td>
<td>19</td>
<td>16</td>
<td>1.17 (0.70–1.82)</td>
<td>36</td>
<td>27</td>
<td>1.33 (0.93–1.84)</td>
</tr>
<tr>
<td>≥75</td>
<td>4</td>
<td>2</td>
<td>1.89 (0.51–4.83)</td>
<td>0</td>
<td>1</td>
<td>0.00 (0.00–5.75)</td>
<td>2</td>
<td>1</td>
<td>4.00 (0.48–14.43)</td>
<td>2</td>
<td>1</td>
<td>2.50 (0.30–9.04)</td>
</tr>
</tbody>
</table>

P value for trend

Follow-up, y

<table>
<thead>
<tr>
<th>Age at diagnosis of prostate cancer, y</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–4</td>
<td>89</td>
<td>68</td>
<td>1.05 (1.05–1.61)</td>
<td>16</td>
<td>18</td>
<td>0.89 (0.51–1.44)</td>
<td>28</td>
<td>18</td>
<td>1.59 (1.05–2.29)</td>
<td>41</td>
<td>29</td>
<td>1.41 (1.02–1.92)</td>
</tr>
<tr>
<td>5–9</td>
<td>49</td>
<td>40</td>
<td>1.22 (0.90–1.61)</td>
<td>11</td>
<td>11</td>
<td>0.98 (0.49–1.76)</td>
<td>14</td>
<td>11</td>
<td>1.32 (0.72–2.21)</td>
<td>22</td>
<td>16</td>
<td>1.36 (0.85–2.05)</td>
</tr>
<tr>
<td>≥10</td>
<td>18</td>
<td>19</td>
<td>0.95 (0.56–1.50)</td>
<td>6</td>
<td>6</td>
<td>0.53 (0.11–1.54)</td>
<td>6</td>
<td>5</td>
<td>1.18 (0.43–2.57)</td>
<td>8</td>
<td>7</td>
<td>1.12 (0.48–2.20)</td>
</tr>
</tbody>
</table>

P value for trend

Table 5. — SIRs and 95% CIs of Colorectal Adenocarcinoma in Other Treatment Cohorts Compared With the General Population (1981–2008)*

<table>
<thead>
<tr>
<th>Age at diagnosis of prostate cancer, y</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>41–64</td>
<td>133</td>
<td>108</td>
<td>1.23 (1.03–1.45)</td>
<td>28</td>
<td>31</td>
<td>1.10 (0.75–1.56)</td>
<td>34</td>
<td>28</td>
<td>1.24 (0.86–1.73)</td>
<td>61</td>
<td>46</td>
<td>1.32 (1.01–1.70)</td>
</tr>
<tr>
<td>65–74</td>
<td>279</td>
<td>219</td>
<td>1.76 (1.56–1.98)</td>
<td>169</td>
<td>123</td>
<td>1.37 (1.17–1.60)</td>
<td>158</td>
<td>111</td>
<td>1.43 (1.21–1.67)</td>
<td>219</td>
<td>167</td>
<td>1.31 (1.15–1.50)</td>
</tr>
<tr>
<td>≥75</td>
<td>379</td>
<td>263</td>
<td>1.44 (1.30–1.59)</td>
<td>148</td>
<td>83</td>
<td>1.79 (1.51–2.10)</td>
<td>90</td>
<td>65</td>
<td>1.38 (1.11–1.70)</td>
<td>116</td>
<td>96</td>
<td>1.21 (1.04–1.45)</td>
</tr>
</tbody>
</table>

P value for trend

Follow-up, y

<table>
<thead>
<tr>
<th>Age at diagnosis of prostate cancer, y</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–4</td>
<td>694</td>
<td>499</td>
<td>1.42 (1.32–1.53)</td>
<td>227</td>
<td>141</td>
<td>1.61 (1.41–1.64)</td>
<td>175</td>
<td>123</td>
<td>1.42 (1.22–1.64)</td>
<td>244</td>
<td>191</td>
<td>1.28 (1.12–1.45)</td>
</tr>
<tr>
<td>5–9</td>
<td>321</td>
<td>239</td>
<td>1.34 (1.20–1.50)</td>
<td>99</td>
<td>71</td>
<td>1.39 (1.13–1.69)</td>
<td>81</td>
<td>61</td>
<td>1.32 (1.05–1.64)</td>
<td>123</td>
<td>91</td>
<td>1.35 (1.12–1.61)</td>
</tr>
<tr>
<td>≥10</td>
<td>82</td>
<td>72</td>
<td>1.14 (0.91–1.42)</td>
<td>22</td>
<td>22</td>
<td>1.00 (0.62–1.51)</td>
<td>26</td>
<td>19</td>
<td>1.40 (0.92–2.06)</td>
<td>29</td>
<td>27</td>
<td>1.09 (0.73–1.66)</td>
</tr>
</tbody>
</table>

P value for trend

that found a strong association between orchiectomy and colorectal adenocarcinoma. The elevated risk (increase in CRC incidence of 30%–40% in men exposed to ADT) was noticeable even after adjust-
ing for potential confounders such as diabetes, obesity, and radiotherapy. Although that study had robust data regarding orchiectomy and GnRH therapy, it included bias from confounding by indication and its participants were older. Our study, which measured relative risk based on a comparison between the study cohort groups and the general population, further demonstrated the findings of the US study.

**Estrogen and Androgen**

We did not find any association between estrogen treatment and colorectal adenocarcinoma. Previous reports, which were based on observational data and results from clinical trials, have suggested that women exposed to exogenous estrogen, including hormone replacement therapy or oral contraceptives, have a decreased risk of CRC. However, the estrogen hypothesis has seldom been evaluated in men.

The relationship between estrogen and androgen is intriguing. Estrogens are produced with androgens as precursors. Progesterone is the first important sexual steroid formed in the body. The androgens (dehydroepiandrosterone, androstenedione, and testosterone) arise thereafter, whereas the estrogens (estrone and estradiol) appear only during the final stage.

Estrogen and androgen may play a similar role in carcinogenesis among women and men, respectively. Estrogen and androgen were postulated to prevent tumor growth by preventing insulin and insulin-like growth factor from binding to their receptors. By contrast, an increased risk of colorectal adenocarcinoma due to androgen deprivation could be possible as the results from animal studies suggest that androgen may have a protective effect on the development of CRC. Androgen receptors have also been found more frequently in the normal — as opposed to cancerous — mucosa in the colon. Thus, orchiectomy or GnRH treatment would directly decrease androgen.

**Specific Subsite**

Our results suggest differences in risk may exist between the proximal and distal colon. The decreasing trend of SIRs associated with adenocarcinoma in the proximal colon over follow-up years could indicate a differing association at that subsite. Select genetic or physiological mechanisms might explain this difference in risk patterns of adenocarcinoma in the proximal and distal colon. The proximal colon originates from the midgut, but the distal colon and rectum originate from the hindgut. Research suggests that different genetic pathways to CRC dominate the proximal and the distal segments of the bowel. These genetic-dependent pathways are influenced by different sex-related factors. Estrogen receptors are distributed differently in the proximal and distal colon. Furthermore, chromosomal instability and microsatellite instability — 2 forms of genetic instability in CRC — mostly occur in the proximal and distal colon, respectively, warranting the genetic basis.

**Limitations**

Strengths of this study include its nationwide and population-based cohort design identified from national registries, its large prostate cancer cohort sizes, and its long and complete follow-up times; however, information on individual prostate cancer treatment was limited. Data on bilateral orchiectomy, which is a common ADT, was available in the patient registry from 1964 onward, but we had no detailed information on other hormonal treatments such as estrogen or antiandrogen medications. We specified the cohorts based on time periods and specific treatments. The results of the sensitivity analyses showed fair consistency with the reported results based on the selected cutoff dates.

Another concern is the introduction of prostate-specific antigen (PSA), which could change the profiles of patients with prostate cancer. PSA screening was introduced in Sweden between 1995 and 2000 and steadily increased from 1% to 10%. Less aggressive prostate cancer might have been diagnosed after the introduction of PSA testing. However, this would have little influence on our results because we stratified the analysis in all 3 subcohorts. Treatments for the first 2 cohorts were defined, whereas influences on the third cohort could have been overlooked. Considering the lower proportion of PSA screening and the large size of this cohort, PSA screening should not engender a substantial influence on the total results.

It is possible that CRC screening might influence the diagnosis of CRC in patients with prostate cancer. In Sweden, CRC screening commenced in the 1980s but patients were limited; the officially organized screening of CRC began in Stockholm in 2008 and in the rest of Sweden in 2014 so CRC screening would not have substantially affected the results of our study. Furthermore, we did not find studies to indicate that colonoscopy was increased in the prostate cancer survivor group in Sweden. It may be possible that patients receiving radiotherapy for prostate cancer may also have had bowel symptoms. Because SIRs were calculated based on a comparison of the prostate cancer cohort with the general population at the same period, such an influence would be minor.

The adverse events of hormone therapy are concerning and may contribute to an increased risk of CRC, but such information cannot be specified in the current study. Further adjustment of obesity and diabetes did not change the association of hormone therapy and CRC in one study.

Lastly, no information was provided on what may
be considered to be confounding factors, including familial background and dietary habits. However, it is unlikely that these factors could explain our results, as it is likely they are equally distributed between patients regardless of prostate cancer treatment.

Although the randomized clinical trial is the gold standard for this type of research, its use was not feasible for our study due to ethical issues.

Conclusions

Results from this population-based cohort study suggest that androgen deprivation therapy, including bilateral orchiectomy, may increase the risk of colorectal adenocarcinoma in men, although confounders of radiotherapy or prostate cancer could not be excluded. Further studies are warranted to elucidate this potential association.

References

Ten Best Readings Relating to Molecular Biomarkers


In this review, the authors discuss the biology of tumor cell dissemination, technical advances, challenges, and potential clinical implications of detecting and characterizing circulating tumor cells.


Aberrant p53 protein expression is associated with an increased risk for neoplastic progression in patients with Barrett esophagus and may be a more powerful predictor of neoplastic progression than a histological diagnosis of low-grade dysplasia.


In this review, the authors describe novel key mutations in myelodysplastic syndromes and their significance in pathophysiology and clinical practice.


The authors describe the genomic landscape of 496 papillary thyroid carcinomas (PTCs). In this study, they observed a low frequency of somatic alterations and extended the set of known PTC driver alterations to include *EIF1AX*, *PPM1D*, and *CHEK2*, along with diverse gene fusions. These discoveries reduced the fraction of PTC cases with unknown oncogenic drivers. The authors propose reclassifying thyroid cancers into molecular subtypes to reflect their underlying signaling and differentiation properties.


The Cancer Genome Atlas study may simplify the classification of thyroid cancer, with the authors suggesting refocused efforts on studying the complexity of the follicular variant of thyroid neoplasia.


An algorithm based on a stepwise analysis with initial immunohistochemistry is presented for ATRX and IDH1-R132H followed by 1p/19q analysis followed by IDH sequencing. IDH sequencing has reduced the number of molecular analyses, leading to a better association with patient outcomes than the World Health Organization classification of 2007.


This review enumerates molecular derangements and targeted agents in soft-tissue sarcoma. Select sarcomas are highlighted to illustrate how pathologists can influence patient care through diagnosis, grading, and molecular characterizations.


This review addresses known recurrent or tumor-specific genetic events in soft-tissue tumors and discusses the molecular approaches commonly used in clinical practice to identify them. Emphasis is placed on the role of molecular pathology in soft-tissue tumor management and the need for pathologists to be familiar with these genetic events.


The authors discuss molecular genetic advances in primary, nonhematological round cell tumors of bone.


The authors compare different methods for determining risk of recurrence in estrogen receptor-positive breast cancer.
*This includes approximately 1,000 oncologists from more than 85 countries.

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CONFERENCE CONTACTS
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