
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
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<sup>2+</sup> molecules, dinucleotides, a polymerase molecule, and a fluorescent-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.\textsuperscript{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.\textsuperscript{14} 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 ($P < .001$).\textsuperscript{16} 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{21}

Researchers have demonstrated that detecting a mutant allele at a ratio of 1:100,000 is possible when using a microdroplet system.\textsuperscript{3,23} 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 al\textsuperscript{24} 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 al\textsuperscript{24} 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.\textsuperscript{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 (PP$_i$) 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 PP$_i$. Because dNTPs are individually added to the reaction, their incorporation can be determined based on the presence or absence of PP$_i$. Therefore, for each dNTP incorporated, an equimolar quantity of PP$_i$ 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 PP$_i$ 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.\textsuperscript{26,27} In 1988, Karas and Hillenkamp\textsuperscript{28} 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 al\textsuperscript{29} 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 Analysis 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 (Clariant, 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 noninvasive 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 research\textsuperscript{68}; 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.\textsuperscript{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.\textsuperscript{71} The company also developed FoundationOne Heme (Foundation Medicine) for hematological malignancies and sarcomas. It is an expanded panel of 405 genes and translocations at 265 loci.\textsuperscript{72} Sarcomas and hematological malignancies are known for the presence of gene fusions that result in the formation of oncogenes that are diagnostic, prognostic, and predictive.\textsuperscript{72} Thus, the results of both tests can be used to inform the health care 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.\textsuperscript{59,73}

**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 tumorogenesis, 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.\textsuperscript{74} Intratumoral heterogeneity in driver mutations and in loci implicated in therapeutic resistance leads to clinical heterogeneity in tumor response to treatment.\textsuperscript{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.\textsuperscript{78} 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.\textsuperscript{9,48,78,79}

Genetic heterogeneity provides the substrate for fueling tumor evolution during tumor progression and therapeutic resistance.\textsuperscript{79} 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.\textsuperscript{80,81}

Tumors may also escape targeted therapy by activating survival pathways such as those observed in BRAF V600E–mutant melanomas treated with vemurafenib.\textsuperscript{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.\textsuperscript{83} In lung cancer treated with EGFR inhibitors, resistance is developed by activation of alternative prosurvival signaling pathways via MET amplification, as well as acquisition of the EGFR T790M mutation, which affects the binding of tyrosine kinase inhibitors.\textsuperscript{74,84}
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.\(^8^5,^8^6\) 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 (eg, presence of mutant \(\text{BRAF}\) 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.\(^8^7\) 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 (\(\text{BRAF}\)) inhibitors in colorectal cancer displaying activating mutations of \(\text{BRAF}\). Although \(\text{BRAF}\) inhibitors appear to inhibit melanomas with \(\text{BRAF}\)-activating mutations, disappointing results were seen in study volunteers with \(\text{BRAF}\)-mutated colorectal cancer.\(^8^8\) This failure has been attributed to the bypass of the \(\text{BRAF}\)-signaling pathway by the EGFR-signaling pathway in colorectal cancer, a pathway not active in melanoma.\(^8^8\) Consequently, health care professionals must use care when using molecular data across tumor types because our knowledge of cancer biology is limited; using single markers may be inadequate to define specific treatment regimens.\(^8^8,^8^9\)

**Variants of Unknown Significance**

With the vast amount of information produced from NGS, molecular pathologists and oncologists are increasingly challenged in managing complex genetic information. A typical large panel analysis can reveal dozens of different mutations, including classical drivers and other alterations of lesser-known consequence. Many of these may be “passenger mutations” or inherited polymorphisms of uncertain consequence. Some, such as inherited germline missense mutations, will result in defective tumor suppressors, such as \(\text{BRCA}\) or \(\text{TP53}\), and may define cancer family syndromes, whereas others may have less certain meaning. Some have suggested that large-panel NGS should be run on both tumor and germline samples from patients to help identify which mutations are associated with somatic tumors and which ones are inherited.\(^9^0\)

**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.\(^9^1-^9^3\) The main challenge facing many laboratories is how to validate the analytical and clinical performance of these advanced tests.\(^9^1,^9^4-^9^6\) NGS technology also poses major challenges for regulatory agencies.\(^9^7\) The rapid adoption of these technologies is outpacing the abilities of governmental agencies to develop effective monitoring strategies.\(^9^8,^9^9\) Cancers are dynamic, heterogenous 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 (eg, conducting a randomized blinded trial for each mutation is not feasible). 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.\(^9^9\)

**Investment Uncertainties**

The implementation of personalized medicine testing has also been hampered by the reimbursement landscape.\(^9^8\) 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
billoning 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


