

Understanding the Landscape of Clinically Available Molecular Testing



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KEYWORDS

- Molecular diagnostics • Precision oncology • Next-generation sequencing (NGS)
- Immunohistochemistry (IHC) • Circulating tumor DNA (ctDNA) • Tumor biomarkers
- Liquid biopsy • Targeted therapy

KEY POINTS

- Molecular diagnostic tests, like other assays provided by medical laboratories, should be selected based on the individual patient's clinical scenario.
- Consider comprehensive genomic profiling (CGP) for rare tumors, when the diagnosis is unclear, and when the clinical presentation is unusual. CGP can also reveal unique combinations of oncogenic drivers in common tumors, aiding in more precise subclassification and personalized treatment selection.
- Recognize that no single molecular test is universally applicable to all clinical situations. Be aware of how pre-analytic variables, such as tissue handling and fixation, can impact assay sensitivity and specificity. Understanding limitations of methodologies can help avoid misinterpretation of test results.

INTRODUCTION

Personalized oncology care is a complex and rapidly evolving field. Traditionally, cancer classification was based on anatomic site, histologic pattern, cytologic features, and staining characteristics observed under the microscope by a trained anatomic pathologist. However, this approach often resulted in significant variation in tumor behavior and therapy responsiveness among patients with the same diagnosis. Cancer is now understood to result from a combination of inherited DNA variants (germline) and accumulated mutations from environmental exposures, replication errors, and aging at the somatic level. These mutations lead to altered gene expression, differentiation, signal transduction, and cell cycle control, among other processes.

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Our growing arsenal of molecular diagnostic assays allows us to include changes in DNA sequence and RNA expression, as well as protein composition, quantity, and cellular compartment location in our tumor categorization. This makes diagnosis more comprehensive and reflective of the underlying biological nature of the disease at the individual patient level. For example, the 2021 WHO Central Nervous System tumor classification system now requires the assessment and integration of molecular findings with anatomic and histopathologic features for a final diagnosis. This includes *IDH1* and *IDH2* gene sequencing, *TERT* promoter methylation, 1p/19q chromosome arm testing for oligodendroglioma and glioblastoma categorization, and methylation signatures to differentiate subtypes of medulloblastomas.¹

Recently, prognostic differences observed between molecular categories (polymerase E-mutated [*POLEmut*], mismatch repair deficient, nonspecific molecular profile, and P53 abnormal [p53abn]) defined by The Cancer Genome Atlas for endometrial cancer analysis have led to changes in the 2023 International Federation of Gynecology and Obstetrics (FIGO) tumor staging for endometrial cancer.² The updated FIGO staging guidelines recommend complete molecular classification in all endometrial cancers for prognostic risk group stratification and as factors that might influence adjuvant and systemic treatment decisions. When molecular classification reveals p53abn or *POLEmut* status in Stages I and II, this results in upstaging to “Stage IICm-p53abn” or downstaging to “Stage IAmpOLEmut” based on worse and better molecularly defined prognosis, respectively. As additional tumor types evolve to more precise diagnoses from molecular feature integration, we aim to improve our ability to apply historical experience and clinical trial treatment responses from patients with more biologically similar tumors. This should aid in predicting clinical outcomes and identifying effective therapies or at least in avoiding biologically implausible agents and their side effects.

CLINICAL SCENARIOS FOR MOLECULAR TESTING

Matching the molecular assay to the stage of the patient’s cancer journey, the available sample types, and the clinical question at hand is crucial in gathering important data to support the shared decision-making process (Fig. 1).

Cancer Detection

Some molecular assays are designed for cancer detection to supplement current screening tests in an asymptomatic, normal-risk population. One example is the polymerase chain reaction (PCR)-based high-risk human papillomavirus (HPV) testing in cervical cancer screening of women 30 to 65 years old, which when negative extends the screening interval to 5 years.³ Another is the multitarget stool DNA test (tests for six *KRAS* point mutations and methylation of *NDRG4* and *BMP3*; Cologuard, Exact Sciences, WI) for colon cancer screening, which when negative in an average risk adult can be repeated every 3 years in lieu of colonoscopy or annual fecal occult blood testing.⁴ In patients with symptoms or radiologic findings highly suggestive of malignancy, but where tissue sampling for diagnosis is risky due to lesion location or the patient’s performance status, or when timely access to tissue sampling services is limited, testing peripheral blood plasma for circulating tumor DNA (ctDNA) can be a pragmatic alternative. This approach may allow patients to avoid or delay invasive procedures if diagnostic or targetable mutations are identified in the blood.⁵

Molecularly Specified Diagnosis, Prognosis, and Misdiagnosis

When a tumor biopsy is obtained, testing the tissue for specific molecular markers can support and refine the probable histopathologic diagnosis. For instance, in breast

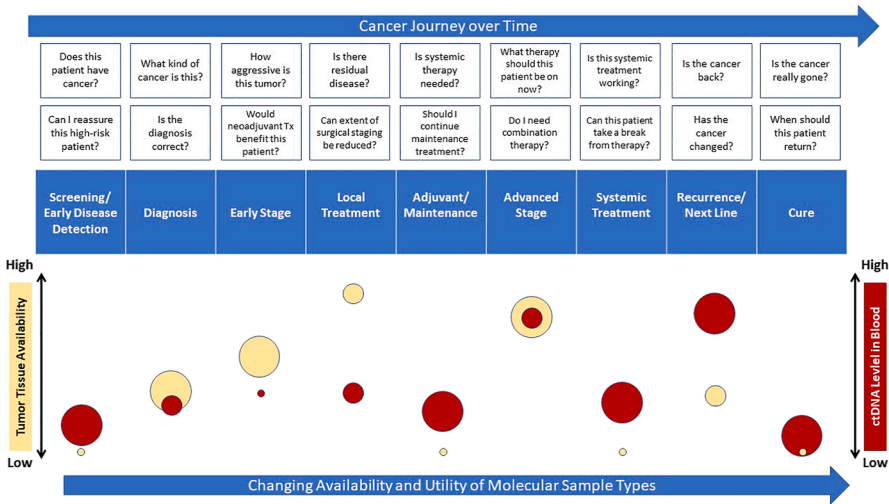


Fig. 1. Timeline of a patient's cancer journey. At each stage of a cancer journey, clinically available molecular assays may provide information to answer evolving clinical questions. The probable availability of tumor tissue appropriate for testing (*orange axis, left*) and the amount of ctDNA present in a patient blood sample (*red axis, right*) also changes at each stage and is reflected by the position of the circle along the vertical axis of graph. The estimated utility of either tumor tissue test (*orange*) or a liquid biopsy (*red*) to answer stage-relevant questions is represented by three different size circles (small circle = low utility; medium circle = moderate utility; large circle = high utility).

adenocarcinoma (BAC) of any stage, routine molecular tests, such as estrogen receptor (ER) and progesterone receptor (PR) immunohistochemistry (IHC), and human epidermal growth factor receptor 2 (HER2) status testing (either via *ERBB2* copy number detection by fluorescent in situ hybridization [FISH] or next-generation sequencing [NGS], or protein expression by IHC), are used to differentiate hormone receptor-positive, HER2-positive, and triple-negative breast cancer (TNBC) subtypes at diagnosis.⁶ Commercially available gene expression profiling assays, such as Prosigna PAM-50 (FDA-approved, 50-gene reverse transcription [RT]-PCR with probe hybridization detection, NanoString Tech, Seattle) and BluePrint and MammaPrint (70-gene mRNA expression level by microarray, Agendia, Irvine, CA), can be used in clinically complex cases to further refine the diagnosis into Luminal A, Luminal B, and basal intrinsic subtypes, as each subtype has different prognoses, risks of recurrence, and likely responses to chemotherapy and hormonal therapies.

In early-stage (node-negative) ER+ or ER-positive BAC, gene signature assays generating expression-based recurrence scores (RS), such as Oncotype DX (quantitative RT-PCR assay of 21 genes) and MammaPrint (microarray-based expression levels of 70 genes), are frequently employed to identify patients less likely to benefit from additional chemotherapy to their hormonal therapy. The OncotypeDX RS was prospectively validated in the TAILORx trial and is now included in National Comprehensive Cancer Network (NCCN) and American Society of Clinical Oncology (ASCO) guidelines for ER-positive, HER2-negative, and lymph node-negative patient management. Currently in node-positive ER-positive BAC, these assays have only prognostic value, but their possible utility in treatment decision-making is under investigation.⁶

Molecular test results are crucial in establishing the correct diagnosis when biologically distinct processes have similar clinical or morphologic presentations. One example where the diagnosis dramatically impacts prognosis and treatment is distinguishing a rare clear cell sarcoma (CCS), typically treated by surgery and radiation, from a more common cutaneous melanoma presenting as a soft tissue mass in an extremity, which may benefit from systemic targeted and/or immunotherapy. Both tumors stain with melanocytic IHC markers, but CCS has a diagnostic fusion involving the *EWSR1* gene, detectable by break-apart FISH or NGS, whereas melanoma may show a high tumor mutation burden (TMB), MEK pathway activating mutations, and an ultraviolet mutational signature (UVsig) from comprehensive genomic profiling (CGP).⁷ A recent study of 8143 tumors with UVsig underscores the potential value of CGP, finding that only 51% had a diagnosis consistent with cutaneous origin (skin and adnexal carcinomas and melanomas) and 34% had a diagnosis without a primary site specified (such as “carcinoma of unknown primary”) where the UVsig may have helped to specify the primary. Most importantly, 13% had a diagnosis of extracutaneous origin, such as lung, salivary gland, non-salivary head and neck, breast and urothelial cancers, and sarcoma, where knowledge of the presence of a UVsig would likely lead to reclassification, restaging, and alternate therapy selection.

Another example of the diagnostic utility of molecular testing and its impact on conventional treatment selection is highlighted by Eskander and colleagues⁸ for distinguishing small cell lung carcinoma (SCLC) and small cell neuroendocrine carcinoma of the cervix (SCNECC) metastatic to the lung. Both can present as aggressive, high-grade carcinomas that stain with neuroendocrine markers, but SCLC is characterized by inactivating mutations in *TP53* (>90%) and *RB1* (>70%) and is responsive to platinum and etoposide chemotherapy. In contrast, more than 85% of SCNECC are positive for HPV16 or HPV18, and nearly half (41%) harbor mutations in the PI3K/AKT/mTOR pathway, and these molecular features may influence first-line and later-line therapy choices.

As molecular information accumulates from testing tumors from millions of patients, additional clinically relevant subtypes of common cancers and new, rare, biologically distinct malignancies are being recognized. This knowledge will continue to impact cancer diagnostic classification and treatment paradigms in the coming decades.

Hereditary Cancer Risk

Inherited genetic changes account for up to 10% of cancer diagnoses. Certain cancer types (such as ovarian, fallopian tube, and peritoneal cancers), early-onset cancers (such as breast, colon, or prostate cancer), multiple sequential cancers, or a strong familial cancer history should prompt consideration of genetic counseling and possible germline genetic testing. Previously, when DNA sequencing was challenging and costly, germline testing typically focused on a few high-yield candidate genes, such as *BRCA1* and *BRCA2*, especially in patients of Ashkenazi Jewish descent. However, when expanded panel testing with greater than 400 cancer-related genes was applied to *mBRCA1/2*-negative families, 44% were found to have deleterious or potentially deleterious variants in other genes.⁹ Because of the higher yield, increased availability, and lower cost associated with NGS assays, large germline panels of hundreds of cancer-related genes have become part of the routine cancer diagnostic workup, even when the suspicion for an inherited cancer syndrome is low. One consequence of expanded testing rather than this setting is the frequent identification of variants of unknown significance rather than those with clear deleterious functional impact. Patients should be counseled about this possibility, as well as supported in understanding and appropriately reacting to indeterminate results. Tumor testing, while

not designed to assess hereditary risk, may detect inherited mutations that are present in both normal and tumor cells, in addition to somatic mutations acquired only by the tumor. Therefore, including trained genetic counselors on molecular tumor boards reviewing tumor molecular testing results can be beneficial for identifying additional patients who may benefit from follow-up germline testing.¹⁰

Treatment Prediction for Molecularly Matched Therapy

A key driver of increased molecular testing is the opportunity to identify patients eligible for targeted therapies, immunotherapies, and antibody-drug conjugates (ADCs). A recent review of FDA oncology drug approvals from 1998 to 2022 found that one-third were classified as precision oncology drugs, requiring genomic profiling for eligibility. More than 45 genes plus two complex genomic signatures, namely microsatellite instability high and TMB high (TMB > 10 mutations/Mb of DNA), were identified as FDA-recognized biomarkers.¹¹ Many additional “tumor type + drug + molecular characteristic” combinations are under investigation in clinical trials or currently being reviewed by the FDA.

This new drug approval paradigm highlights the importance of quality and regulatory oversight in molecular diagnostic tests. An FDA-approved companion diagnostic (CDx) is an in vitro diagnostic (IVD) that guides the safe and effective use of a particular therapy and is often a key factor in treatment decisions.¹² To achieve this designation, a diagnostic test developer performs and submits extensive validation studies, often including hundreds or even thousands of samples, to demonstrate analytical performance and clinical validity, and its use in a therapeutic clinical trial establishes clinical utility. When a CDx assay is performed following the manufacturer’s technical specifications, any laboratory should be able to achieve comparable analytical performance for the intended use. For this reason, when a laboratory adds a new FDA-approved IVD to its menu, they only need to perform a verification on a set of positive and negative control samples, rather than performing the more extensive analytical validation themselves. IVDs and CDxs result in standardization between laboratories, but limit flexibility, such as accepting alternative specimen types or using alternative reagents or instrumentation. In contrast, laboratory-developed tests (LDTs) can be used for clinical decision-making if they are performed in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory and have been approved by the laboratory director for clinical use after the performance of a complete validation according to laboratory accreditation body standards, such as ISO 15189, the American Society of Clinical Pathology, or the College of American Pathologists. Many LDTs are well-designed, well-validated, and accurate. However, LDTs performed by different laboratories to test for the same biomarker, may use different methodologies, reagents, instrumentation, and positive and negative cutoffs, and thus may yield different results on the same patient sample. Inter-laboratory sample exchange and biannual proficiency testing for each assay are employed to minimize differences in test results between LDTs run in different laboratories.¹³

Treatment Response Monitoring and Identification of Resistance Mechanisms

Blood-based, ctDNA assays can be used after diagnosis and alongside radiographic imaging to molecularly monitor treatment response, assess minimal residual disease (MRD), or detect early recurrence.¹⁴ Digital droplet PCR (ddPCR) for the *BRAF* V600 E mutation characteristic of hairy cell leukemia has been used to assess MRD in this disease since 2016. More recently, tissue-informed ctDNA treatment response monitoring (TRM) assays, in which an antecedent tumor-specific mutational profile is used to select a fixed or build a custom PCR assay, have become clinically available

for solid tumors. Examples of this type of test include FoundationOneTracker (Foundation Medicine Inc, MA) and Signatera (Natera, CA), which use a targeted CGP cancer panel or whole exome sequencing tissue baseline to identify 2 to 16 trackable somatic variants and build a patient-specific, highly sensitive PCR assay that measures variants in the plasma at an allele frequency as low as 0.01% (equivalent to one mutant haploid genome in a background of 10,000 normal haploid genomes). In early clinical studies of these assays, decreases of trackable variants correlated with subsequent clinical and radiologic responses to therapy in lung, colon, and breast cancer.^{15–17} On recurrence, or when cancer progresses on treatment, molecular profiling performed on a posttreatment specimen can identify additional, acquired mutations causing resistance to therapies. For example, the *EGFR* T790M mutation which conveys resistance to second-generation *EGFR* inhibitors and *ESR1* ligand binding site mutations that develop in some patients with breast cancer on hormone therapy can be detected in both tissue samples and ctDNA. In this setting, liquid biopsies are extremely valuable, enabling many advanced stage patients to avoid the risk and discomfort of another invasive biopsy procedure.^{18–20} Additionally, as patients are diagnosed earlier, survive longer, or appear cured, molecular tests also become crucial for differentiating a second primary malignancy from a recurrence. In turn, this distinction can impact prognosis, management, clinical trial eligibility, and familial risk stratification.

SELECTING THE OPTIMAL MOLECULAR ASSAY

Many factors influence the selection of a molecular assay for a specific clinical question. Key considerations include the assay's size and scope, the patient sample type, the molecular target, and the specific assay methodology.

Assay Scope: Narrow Versus Broad Testing Approaches

The molecular test landscape includes assays that interrogate a single biomarker, those testing multiple biomarkers relevant to a specific tumor type, and pan-tumor assays assessing a wide range of biomarkers. Single-analyte and narrow panels may be preferred for their availability, speed, lower tissue requirements, or cost. In well-characterized cases with few treatment options, single marker tests or small hotspot panels might be appropriate, at least initially. Examples include PCR-based assays for *EGFR*, *ALK*, and *KRAS* in a lung biopsy with non-small cell lung cancer (NSCLC), mismatch repair IHC on a colorectal carcinoma surgical resection, or HER2 FISH on small gastric carcinoma biopsies. While focused assays are suitable when the diagnosis is accurate, and the clinician has a clear hypothesis about the relevant information needed, if the patient has been misdiagnosed, the limited additional molecular information may be misleading and compound the error.

Broad CGP assays, not limited to common mutations for a particular tumor, reveal the unique “snowflake profile” of oncogenic drivers in each patient. Pan-tumor CGP assays are less sensitive to misdiagnosis and may help reveal a more accurate diagnostic picture, and have tissue-derived DNA or cell-free DNA (cfDNA) input requirements and costs comparable to 5 to 10 single-analyte tests. Moreover, CGP is efficient and economical for identifying low-frequency, highly actionable alterations, such as *NTRK* or *RET* fusions found in less than 1% of all cancers.²¹ CGP is particularly helpful for rare tumors, such as cholangiocarcinoma or gastrointestinal neuroendocrine carcinomas, in identifying mutation-matched clinical trials or FDA-approved therapies. For example, anti-RET tyrosine kinase inhibitors were first approved in lung and thyroid carcinomas. Selpercatinib's expanded approval for any

RET-mutated cancer was based on a basket-type clinical trial, with many patients identified by CGP.²² This trial outcome has made testing for *RET* fusions important regardless of the tumor type.

Patient Sample Source: Tumor Tissue Versus Peripheral Blood

Tumor tissue and blood are the most commonly used sample types for molecular testing. The choice between them depends on various factors including availability, timing of testing, treatments received, the extent and location of the disease, patient performance status, and care goals (see Fig. 1). Techniques for assessing DNA, RNA, and proteins from formalin-fixed paraffin-embedded (FFPE) tissue samples have improved significantly over the past 20 years. Archival diagnostic biopsies, needle aspirations, and surgical resections often provide a ready substrate for molecular analysis without the risks associated with obtaining fresh tissue. Cell blocks from pleural and peritoneal effusions and direct cytology smears made during rapid onsite assessment of minimally invasive biopsy procedures can also be used for tissue-based molecular assays and are sometimes overlooked as a potential option. It is important for pathologists, proceduralists, and treating physicians to be mindful of tissue utilization at diagnosis, especially when biopsy material is limited, to enable future testing options.

When a suitable tissue sample is not available—for instance, if the diagnostic biopsy has been exhausted, exposed to an incompatible fixative, or lost—testing a blood sample for ctDNA becomes a practical alternative. The plasma component of peripheral blood contains fragments of cfDNA shed from dying cells throughout the body, including normal tissues, bone marrow, and white blood cells.²³ In about 75% of advanced cancers, a small amount of ctDNA is also present. The amount of ctDNA increases with later-stage, higher disease burden, untreated conditions, or progression on therapy. However, even early-stage tumors, especially in high shedding types such as small cell lung, liver, colorectal, bladder, and breast cancers, may have detectable ctDNA.

Unlike tissue testing where tumor nuclei amount can be visually estimated, the ctDNA fraction of total cfDNA is often less than 1% and cannot be predetermined before testing. If a liquid biopsy shows no alterations, it could either accurately reflect an absence of mutations in the tumor, or it could be due to ctDNA levels below the assay's detection limit, resulting in a false negative.²⁴ Some recent assays quantify the tumor fraction in cfDNA, using aneuploidy and maximum mutant allele frequency as indicators, to gauge the reliability of a negative result. With a ctDNA tumor fraction (%TF) cutoff greater than 1%, the concordance between mutations detected in tissue and concurrent liquid biopsy samples is over 90%. However, this concordance drops below 40% when %TF is less than 1%.²⁵ Interestingly, ctDNA %TF itself is emerging as a prognostic biomarker in several tumor types, with higher pretreatment percentages associated with poorer prognosis. Conversely, a significant drop in %TF or an undetectable level posttreatment %TF correlates with more substantial radiological changes and a more durable response to therapy.^{26,27}

An important complexity associated with liquid biopsy is detection of mutations that arise from clonal hematopoiesis (CH) in the bone marrow, rather than from tumor or germline. CH-associated mutations do not predict tumor responsiveness to targeted therapy and can confound ctDNA TRM applications. CH-associated variants in plasma are very common, with some studies showing they are present in ~25% of liquid biopsy samples and account for up to 50% of identified mutations.²⁸ When some ctDNA variants in a sample from a patient with a solid tumor occur in myeloid malignancy-associated genes, such as *DNMT3A*, *TET2*, and *ASXL1*, it is more obvious

that they are either of CH origin or less commonly indicative of a synchronous myelodysplastic syndrome, myeloproliferative neoplasm, or unrecognized leukemia. Some genes, such as *TP53*, are commonly altered in both solid tumors and CH. One study of paired tumor tissue and peripheral blood mononuclear cells (PBMCs) found up to 15% of *TP53* mutations thought to be tumor-derived were instead identified in the PBMC sample only. Less frequent, but more clinically impactful, are targetable genes which are typically tumor-associated, but may also infrequently occur in CH.²⁹ One study found that 10% of a prostate cancer cohort had mutations in homologous recombination repair genes associated with PARP inhibitor responsiveness but these were CH-derived rather than originating in their prostate tumor. Another study found 3% of patients with lung cancer had CH-derived *KRAS* variants that could suggest lack of other targetable mutations if lung tumor-derived. Misinterpretation of CH mutations as drivers of a patient's solid tumor could lead to the use of inappropriate, expensive, and potentially toxic therapies. Efforts to improve liquid biopsy technology, such as fragmentomic analysis and paired buffy coat sequencing, are showing promise in differentiating CH and enhancing liquid biopsy specificity.

Molecular Targets and Impact of Sample Quality to DNA, RNA, and Protein-based Assays

DNA analysis in tumor cells allows for the identification of mutations, including base substitutions, insertions or deletions of nucleotides, translocations, and changes in gene copy number. DNA is relatively stable, with a predictable number of chromosome copies in normal cells (typically 2, but up to 4 in cells undergoing mitosis or in normally tetraploid hepatocytes). However, because only about 1% of human genomic DNA is coding (exons) and less than 10% of that is considered cancer-relevant, enrichment strategies are necessary. These strategies focus sequencing resources on high-yield regions, such as exons of cancer-related genes. One such method, hybrid capture, uses synthetic oligonucleotides or “baits” complementary to sequences within genes of interest. Biotin on the baits allows patient DNA-bait hybrids to be captured by streptavidin beads for sequence analysis, whereas unbound DNA fragments are washed away.

Rearrangements often occur within non-coding regions and are challenging to detect via targeted DNA sequencing. Because intronic sequences between coding exons are removed by splicing during gene transcription, RNA-based assays will detect inappropriately adjacent coding sequences, making them more sensitive for gene fusions. In addition, the presence of the fusion transcript itself in the tumor cell provides evidence supporting functional significance.

Finally, most DNA mutations must be translated into aberrant proteins to affect cell function, so proteins are potentially better biomarkers because they are closer to cellular function. However, since currently most clinically available protein assays in oncology, such as IHC, are limited to analyzing one protein at a time, analysis of multiple expressed proteins may not be possible when tissue is limited.

Variability in sample quality due to pre-analytic factors such as biopsy method, fixative type, fixation duration, exposure to low pH, sample age, and storage conditions can impact DNA and RNA yields and quality. These factors may also cause conformational changes in proteins, altering antibody binding in IHC. Adhering to tissue handling best practices is crucial for the success of many molecular tests and precision medicine.³⁰ Tumor samples always contain a mixture of tumor and normal cells. For assays testing an aggregate of extracted DNA or RNA, the proportion of tumor nuclei (%TN) affects sensitivity. Estimating %TN through microscopic examination

and using enrichment techniques to increase %TN are critical for optimizing assay performance.³¹

METHODOLOGIES

Common DNA Analysis Methodologies

Common methodologies in DNA analysis include in situ hybridization (ISH), PCR, and NGS.

In situ hybridization

ISH detects rearrangements, amplifications, and losses at the individual cell level using DNA probes complementary to the target gene segment. FISH, a variant of ISH, uses fluorescently tagged probes, visible under a fluorescence microscope. In gene amplification or loss assessments, the number of fluorescent signals from the gene probe binding is counted and compared with signals from a centromeric reference probe to determine gene copy number. This process distinguishes specific gene amplifications from overall chromosomal gains. For instance, patient responses in trastuzumab clinical trials for breast cancer and the Vysis HER2 FISH CDx established thresholds for both absolute *ERBB2* copy number and the *ERBB2* gene to chromosome 17 centromere ratio.⁶ This threshold predicts benefit and has become the benchmark for clinically significant gene amplification.

Polymerase chain reaction in oncology

PCR is a molecular technique widely used in oncology for the sensitive, specific, and rapid amplification of nucleic acid sequences.³² It can detect genomic alterations in a single gene or serve as a preliminary step for broader analysis. The standard PCR process involves cycles of temperature changes, target-specific forward and reverse oligonucleotide primers, and a heat-stable DNA polymerase that duplicates the target sequence. Each cycle doubles the amount of targeted DNA, leading to exponential amplification.

PCR versatility extends to multiplex PCR, which targets multiple genes simultaneously, and reverse transcriptase PCR, utilized for analyzing RNA transcripts from oncogenes or fusion genes. Real-time PCR combines amplification with simultaneous detection and quantification of the PCR product through fluorescence measurement, and can provide both qualitative and quantitative data on mutant versus wild-type sequences. Digital droplet PCR (ddPCR) further refines this process, enabling more precise quantification of low-abundance nucleic acids. This precision is invaluable in assays for MRD and TRM. However, PCR limitations include the potential to miss alterations outside the amplified regions or within primer-binding sequences, and false positives due to amplification errors that mimic tumor mutations.

Next-generation sequencing

NGS uses “sequencing by synthesis,” a more advanced method than the earlier Sanger sequencing chain termination approach.³³ NGS is faster and more cost-effective per base pair sequenced, allowing for whole genome sequencing within a clinically relevant timeframe. In NGS, millions of short, single-stranded DNA fragments representing overlapping sections of the genes of interest are arrayed on a small glass flow cell surface. During sequencing, fluorescently labeled nucleotides (G, A, T, or C) are introduced sequentially. These nucleotides bind only to complementary sites at the end of the growing fragment. A high-resolution camera captures images of the fluorescent signals across the flow cell, which are compiled to reconstruct each DNA fragment's sequence.

Sophisticated computational algorithms are used in NGS to assemble the overlapping fragments of DNA sequence into entire genes. They also determine the depth of coverage, which is the frequency at which each nucleotide position is sequenced, identify deviations by comparing them to a known reference sequence, and establish the variant allele frequency (VAF). VAF is calculated by dividing the number of reads containing the variant by the total reads at that position. High unique depth of coverage is crucial for assay sensitivity, as it enables true mutations to be detected even when diluted by DNA from non-tumor components in tissue of cfDNA testing.

RNA Analysis and Sequencing

RNA sequencing (RNAseq), a technique similar to DNA sequencing, can provide important complementary information about a patient's tumor. RNA yield from different cell types is more variable than DNA, and the number of copies of each RNA varies widely based on its transcription level and stability. Gene expression of total RNA (which includes coding and noncoding messenger, ribosomal, and transfer RNA) or purified mRNA (ie, polyA RNA selected by oligo(dT)-coated beads), can be analyzed by hybridization to expression microarrays or by whole transcriptome RNA-seq (WTS).³⁴ For RNAseq, reverse transcriptase PCR converts mRNA into a complementary, full-length, single-stranded DNA (cDNA). In WTS, this cDNA library is sequenced in its entirety. When focus on a subset of genes of interest is desired, the cDNA library can be enriched by hybrid capture or amplicon-based methods. RNAseq is capable of identifying the relative abundance of transcripts, splice variants, post-transcriptional modifications, and gene fusions within tumor tissue, which can provide additional insights into the unique biology of the patient's cancer.

Because RNA is degraded rapidly by ubiquitous RNases in tissue until they are inactivated by fixation, prompt placement of tissue in 20% neutral-buffered formalin is a must for successful RNA testing. In addition, RNAseq is even more sensitive than DNAseq to other pre-analytic variables, with prolonged tissue fixation times, exposure to low pH conditions, older sample age, suboptimal storage conditions, and handling of unstained cut sections reducing RNA yield and quality from a given sample. Unfortunately, these factors may result in some tissue samples yielding less RNA than assay input requirements or poor sequencing performance reflected in sample-specific quality control metrics and contribute to a higher RNAseq assay failure rate.

Immunohistochemistry

IHC has been a cornerstone in molecular diagnostics and precision medicine since the early 2000s. It plays a pivotal role in detecting specific proteins, such as ER in breast cancer, which guides hormonal therapies such as exemestane and fulvestrant. Similarly, detecting tyrosine-protein kinase KIT (c-KIT) overexpression in gastrointestinal stromal tumors has been critical for identifying candidates for imatinib therapy. IHC involves using antibodies to target antigens within FFPE tissue sections. The primary antibody binds to the target antigen, and a secondary antibody often linked to a fluorescent tag or an enzyme that catalyzes a chromogenic reaction, is used to detect the primary antibody. This method amplifies the signal, enhancing sensitivity and allowing for a wide variety of primary antibodies. A contrasting counterstain aids a pathologist in interpreting the staining pattern. Scoring IHC can range from a simple "positive," "negative," or "equivocal" qualitative result to a more complex semiquantitative assessment based on staining intensity and the proportion of positive tumor cells.

Programmed cell death ligand 1 immunohistochemistry complexity

Programmed cell death ligand 1 (PD-L1) IHC exemplifies the intricacies encountered when different primary antibody clones and scoring systems are developed for various anti-PD-L1 immunotherapies across cancer types.³⁵ In NSCLC, the Dako PD-L1 IHC 22C3 pharmDx assay employs a tumor proportion score (TPS), which is the percentage of tumor cells showing any intensity of membranous staining. A TPS over 1% is a criterion for pembrolizumab immunotherapy. The same 22C3 assay, using a combined positive score, is applied in cervical cancer, head and neck squamous cell carcinoma, esophageal squamous cell carcinoma, and TNBC, with varying thresholds for pembrolizumab eligibility. Other clones, such as PD-L1 28-8 and Ventana PD-L1 SP142, are used for different therapies in NSCLC and other cancers, each with unique scoring systems and thresholds. When only a single, generic PD-L1 option is listed on a test menu, it may mean only a single clone is available or that the laboratory will select the most appropriate clone and scoring system from those they offer based on the clinical diagnosis provided. Because these nuances can be crucial for guiding treatment decisions, clarifying your laboratory's approach and discussing cases with uncertain or rare diagnoses with the pathologist is recommended.

Limitations of immunohistochemistry

IHC faces several limitations, including complexities of multiple antibody clones for the same protein for use across the same and different tumor types, intricate scoring systems, the limited availability of reliable multiplex assays, interobserver variability in stain interpretation, and potential pre-analytic variables affecting assay sensitivity and specificity. These limitations have spurred a shift toward nucleic acid-based biomarkers, yet IHC remains a vital tool due to its accessibility, cost-effectiveness, and quick turnaround. Additionally, new IHC CDx assays for immunotherapy agents and ADCs are continuing to be developed and integrated into clinical practice.

SUMMARY

In oncology, the past 3 decades have witnessed a significant shift from the supremacy of the histopathologic diagnosis toward molecularly informed, individualized tumor classification, laying the foundation for more personalized cancer treatment. Advances in technologies such as PCR, NGS, and IHC have led to widely available, clinically validated molecular tests, which are deepening our understanding of cancer at the molecular level and unlocking patient access to an increasing number of targeted treatment strategies. Clinicians now have a variety of tools to diagnose cancer and differentiate new subtypes, predict effective therapies, monitor treatment response, detect emergence of treatment resistance, and an opportunity to choose the molecular approach that fits the individual patient-specific clinical situation. Although integrating these molecular insights into clinical practice can be complex, the overarching aim is clear: to improve patient outcomes by making informed, personalized treatment decisions based on each patient's unique cancer molecular profile.

CLINICS CARE POINTS

- Pursue a Personalized Approach to Molecular Testing: Tailor the selection of molecular diagnostic tests to the individual patient's clinical scenario, considering the specific clinical question, disease stage, available samples, prior treatments, overall health status, and care goals. Choose the test that is most likely to be technically successful and will provide the most actionable and relevant information.

- **Understand Limitations of Different Methodologies and the Impact of Pre-analytic Variables of Assay Performance:** Recognize that no single molecular test is universally applicable to all clinical situations. Be aware of how pre-analytic variables, such as tissue handling and fixation, can impact sensitivity and specificity. Understanding limitations can help avoid misinterpretation of test results.
- **Interpret Liquid Biopsy Results Carefully:** Liquid biopsies have a significant potential for false negatives, especially when the circulating tumor DNA (ctDNA) fraction is below the assay's detection limit. Variants from clonal hematopoiesis can be misinterpreted as tumor-derived leading to clinical false positives. High levels of ctDNA can be associated with worse prognosis.
- **Comprehensive Profiling for Diagnostic Clarity and Rare Tumors:** Consider comprehensive genomic profiling (CGP) for rare tumors or when the diagnosis is unclear. CGP can reveal unique "snowflake profiles" of oncogenic drivers, aiding in accurate diagnosis and treatment selection.

DISCLOSURE

J.A. Elvin is an employee of Foundation Medicine, Inc, a wholly owned subsidiary of Roche Holdings, Inc and Roche Finance Ltd; this employee has equity interest in an affiliate of these Roche entities.

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