MOLECULAR

Advancing Toward Personalized Medicine in Lung Cancer:

A Focus on ALK Gene Rearrangement Testing

Abbott

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INTRODUCTION—NSCLC AND ALK

In the last decade, advances in genomics and molecular biomarker testing have led to tremendous improvements in oncology practice. Today, physicians have access to better prognostic information and more effective, targeted therapies for cancers that are considered to be minimally responsive to treatment with traditional chemotherapies, the historical standard of care. Technology is helping to drive many of these advancements: physicians now have access to more detailed information about their patients with shorter turnaround times for tests, and patients are more informed about their disease and treatment options. These advances are ushering in a new era of personalized medicine.

Lung cancer has benefited considerably from this revolution in genetic testing. Prior to 2011, a lung cancer diagnosis was often considered a "death sentence," and traditional staining techniques were used to differentiate between the less common small cell lung cancer (SCLC) and the more common non-small cell lung cancer (NSCLC) and their subtypes. Although there was a differentiation between squamous cell carcinoma and adenocarcinoma, the most common identified histotypes of NSCLC, a standard cocktail of chemotherapy was recommended across all NSCLC subtypes.[1] But in the 2000s, this began to change when researchers identified several actionable genetic targets in lung cancer tissue that could offer prognostic information or predict treatment response, including the epidermal growth factor receptor (EGFR) and Kirsten rat sarcoma (KRAS) genes.[2] In 2007, the rearrangement of the anaplastic lymphoma kinase (ALK) and EML4 genes produced a tyrosine kinase that was shown to be another plausible target for NSCLC therapy.[3]

The industry ushered in a new era in 2011 with Pfizer's release of a new tyrosine kinase inhibitor (TKI), Xalkori® (crizotinib), for the treatment of NSCLC in patients with tumors positive for ALK gene rearrangements, which markedly increased survival in this patient population. For the first time, the Food and Drug Administration (FDA) approved a drug for NSCLC, Xalkori® (crizotinib), which was codeveloped with its companion diagnositc (CDx) assay, the Vysis ALK Break Apart FISH Probe Kit (Abbott); this CDx was designed to detect ALK gene rearrangements using fluorescence in situ hybridization (FISH). Pfizer selected FISH technology due to the high sensitivity of this test in identifying cases positive for ALK gene rearrangements, some of which were missed using immunohistochemistry (IHC) or reverse transcriptase polymerase chain reaction (RT-PCR) during the clinical trial.[4] The excitement over this new target for therapy inspired further research into personalized medicine for lung cancers and the hunt for more actionable targets. Since 2011, multiple additional targets in NSCLC cancers, with associated therapies, have continued to be identified, including the BRAF gene mutation and ROS1 gene rearrangements.

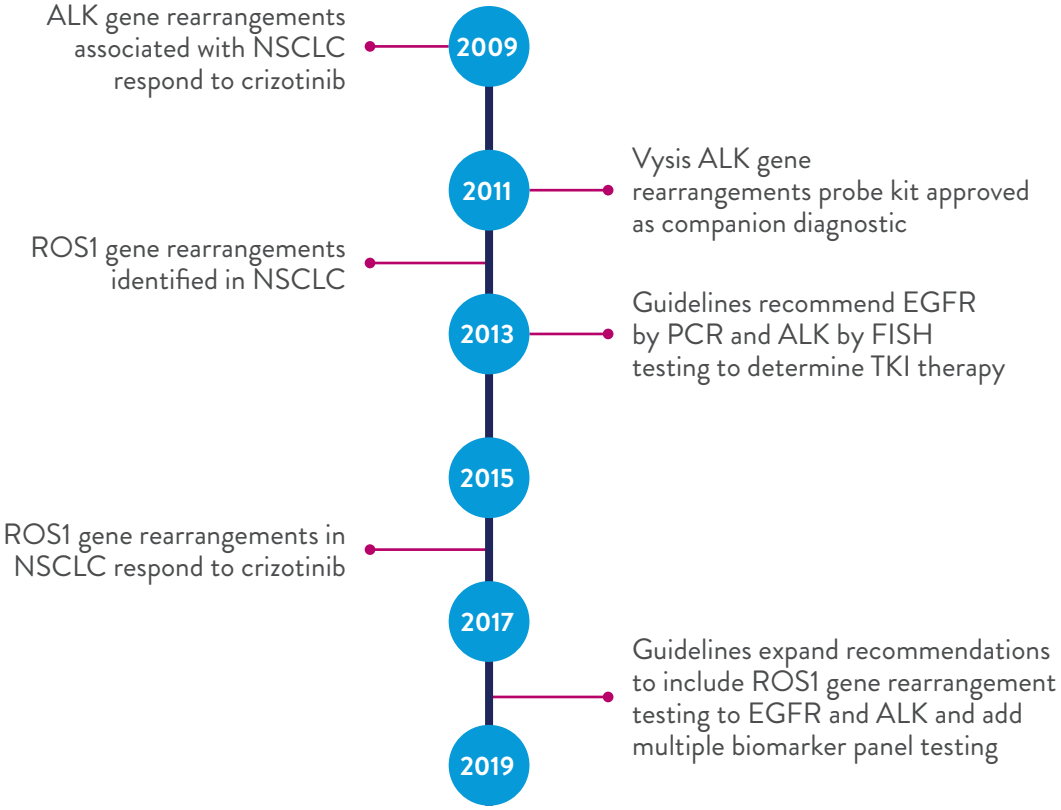
In response to the expanding role of molecular testing, the College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC), and the Association for Molecular Pathology (AMP) released the first molecular testing guideline in 2013 for selection of lung cancer patients for EGFR and ALK TKIs. This document defined the best practices in molecular testing for therapy selection in advanced-stage NSCLC patients and specifically recommended testing patients for EGFR mutations and ALK rearrangements.[5] For ALK testing, the guidelines recommended that

For the first time, the Food and Drug Administration (FDA) approved a drug for NSCLC, Xalkori® (crizotinib), which was codeveloped with its companion diagnostic (CDx) assay, the Vysis ALK Break Apart FISH Probe Kit (Abbott).

laboratories use a dual-labeled break apart ALK FISH probe to select patients for TKI therapy; alternatively, if carefully validated, ALK IHC could be considered as a screening methodology for selecting specimens for ALK FISH testing.[5] A year later, the American Society of Clinical Oncology (ASCO) issued guidelines reaffirming these recommendations and suggesting that if an institution was not using FDA approved assays, that it should develop an institution-specific testing algorithm, where IHC can be used as a screening method, followed by the FDA approved Vysis FISH ALK test for confirmation.[6]

Today, as the list of drugs and CDx tests that target specific mutations and biomarkers continues to expand, it is more evident than ever that personalized medicine is rapidly becoming the standard of care for patients with certain kinds of cancers. But sorting through the testing procedures, which are constantly changing and evolving, for each patient can easily become overwhelming for clinicians. As such, guidance is needed about the optimal practices for disease diagnosis and therapy response monitoring in this new age of personalized medicine. This review focuses on the role of current and emerging technologies for ALK testing in NSCLC and provides an overview of the guidelines, recommendations, and common practices being followed throughout the world to guide healthcare professionals in the choice of technologies in NSCLC diagnostics.

FIGURE 1: ADVANCEMENTS TOWARDS PERSONALIZED MEDICINE IN NON-SMALL CELL LUNG CANCER



SLIDE-BASED TESTING FOR ALK

Initial studies looking at ALK gene rearrangements were performed on NSCLC tissue formalin-fixed paraffin-embedded (FFPE) sections using ALK break apart FISH probes, as this technology demonstrated high sensitivity and specificity in identifying the ALK gene rearrangements. At the time, RT-PCR and IHC tests were not as reliable as FISH for identifying patients who would benefit from crizotinib therapy.[4] However, subsequent investigations were designed to identify other methodologies for testing ALK gene rearrangements, searching for an IHC equivalent to the FISH gold standard for identifying ALK-positive NSCLC patients. Ultimately, two antibody clones were identified and commercialized as diagnostic tests: the Ventana ALK (D5F3) CDx (IVD, CE) and the Novocastra Mouse Monoclonal Antibody p80 (ALK) (5A4 clone) (CE). The Ventana (D5F3) CDx test requires the use of the OptiView DAB IHC Detecting Kit and the OptiView Amplification Kit to improve the detection process along with automated processing with the BenchMark XT Immunostainer. This CDx test also requires result interpretation (the evaluation of staining patterns) to be based on a binary scoring system (positive or negative) instead of using a more standard four-tier scoring system (0 to 3+) for IHC analysis.[7] In contrast, the Novocastra (5A4) does not have specific detection kit requirements, and the staining patterns are assessed with the more common four-tier scoring pattern, but the results are still affected by the potential for subjective estimation of staining. These strict processing requirements, coupled with the variation in result interpretation, introduce the possibility for inconsistent results with the two IHC assays. In contrast, the clinically validated 15% positive cell cutoff for the Vysis ALK Break Apart FISH Probe Kit was established during the crizotinib clinical trial and did not require a supplemental interpretation guide, such as the one released for the Ventana ALK (D5F3) CDx.[8] Moreover, the FISH test was later FDA-approved to be performed either manually or using the VP2000 Processor automated processor.[9]

With the availability of both FISH and IHC-based ALK testing, the optimal approach to molecular testing has been unclear. A 2016 analysis of 12 different studies evaluated testing methods with almost 4000 NSCLC specimens in locations throughout the world.[10] The authors concluded that using IHC to screen NSCLC cases was a cost-effective method, given that a four-tier scoring pattern (such as recommended for the Novocastra ALK test) was employed, and the 1+ and 2+ staining levels would be reflexed to ALK FISH analysis. This study, along with several others comparing IHC to FISH using the two leading clones (5A4 and D5F3), found that in most cases, IHC was either not as sensitive or specific as FISH[7,11–15] (See Table 1 on page 6), including the studies conducted for FDA approval of the Ventana D5F3 IHC assay.[20] (See Table 2 on page 6). These studies and others demonstrate a range of concordance between IHC and FISH, likely due to some of the drawbacks of IHC, including lack of internal controls and consistency in methodology. Therefore, if IHC is used to screen cases, caution should be used in the interpretation of results, especially when used to select therapy. In these cases, confirmation by FISH is recommended.

Although both FISH and IHC technology revolve around the processing of samples onto slides, the FISH technology may be perceived as more complex because it requires specialized fluorescence microscopes and extended training for the performance and assessment of the samples. A recent study compiled data from three clinical trials to assess the 15% cutoff that was established for the Vysis ALK Break Apart FISH Probe Kit.[16] The

Studies have also shown that IHC ALK testing appears to have a greater risk of false-positives than FISH, and quality control related to staining in IHC can also be a problem.[18] Overall, the lower sensitivity with IHC is probably the most significant limitation when comparing IHC with FISH.[19]

researchers found that this cut-off was still supported for clinical utility in identifying patients who would benefit from crizotinib treatment, thus validating the clearly defined interpretation criteria for FISH testing. The inconsistency in scoring recommendations between the two leading IHC assays and the need for scoring guides demonstrates that IHC, like FISH, requires training for proper evaluation.[17] Studies have also shown that IHC ALK testing appears to have a greater risk of false-positives than FISH, and quality control related to staining in IHC can also be a problem.[18] Overall, the lower sensitivity with IHC is probably the most significant limitation when comparing IHC with FISH.[19]

TABLE 1. SENSITIVITY AND SPECIFICITY OF IHC CLONES FOR ALK, COMPARED TO GOLD STANDARD FISH FOR ALK GENE REARRANGEMENTS, META-ANALYSIS OF VARIOUS STUDIES[7,11–15]

CLONE	NO. OF CASES	SENSITIVITY	SPECIFICITY
5A4[11,13–15]	735	100%	96.2%
	594	100%	98.1%
	72	96%	100%
	303	90%	99.3%
	1,031	90.9%	98.3%
Combined Studies	**2,592**	**94.3%**	**97.8%**
D5F3[7,12–15]	99	83.3%	100%
	231	93.9%	100%
	594	100%	99%
	72	96%	100%
	1,031	90.9%	99.8%
Combined Studies	**2,008**	**93.3%**	**99.6%**

TABLE 2. AGREEMENT RATES BETWEEN VENTANA ALK (D5F3) CDx ASSAY AND VYSIS ALK BREAK APART FISH PROBE KIT IN TRIAL 2[20]

AGREEMENT RATES BETWEEN ALK ASSAYS	POSITIVE % AGREEMENT (95% CI)	NEGATIVE % AGREEMENT (95% CI)	OVERALL % AGREEMENT (95% CI)
VENTANA ALK (D5F3) CDx Assay and Vysis ALK Break Apart FISH Probe Kit	92.7% (88.2–95.6%)	94.8% (92.2–96.6%)	94.1% (92.0–95.8%)

TESTING METHODOLOGIES EVOLVE IN NSCLC

As molecular analysis to identify targeted therapies for patients with NSCLC is becoming the standard of care, the exact method and approach to this analysis is still evolving. A reassessment and update to the guidelines from CAP, IASLC, and AMP in 2018 added ROS1 rearrangements as a target for ALK tyrosine kinase inhibitors, resulting in three molecular targets required for testing upon initial diagnosis of NSCLC.[21] Specifically, the guidelines stated that testing for ROS1 gene rearrangements is to be performed by FISH methodology and EGFR mutation by PCR. For assessment of ALK gene rearrangements, the update included cytologic preparations as suitable specimens for testing (in addition to cell blocks).[21] The new guidelines also stated that a properly validated IHC method was an acceptable alternative to FISH, asserting that the evidence supporting the use of IHC was adequate when the IHC results are clearly positive. But in scenarios with challenging to interpret cases, the test should be followed up with another test such as ALK FISH.[21] Until recently, a similar recommendation was maintained by the National Comprehensive Cancer Network (NCCN) guidelines specific to testing for ALK gene rearrangements with IHC, where secondary confirmation by FISH was encouraged.[22] The current guidelines now state that IHC can be deployed as an effective screening strategy, or the FDA-Approved IHC (ALK [D5F3]) CDx Assay can be utilized as a stand-alone test.[23] The new NCCN guidelines also acknowledge that other technologies, like next generation sequencing (NGS), can detect ALK fusions. Yet, they do not advocate for any specific NGS assay, explicitly stating that not all types of alterations are detected by individual NGS assays, implying that a combination of assays may be necessary.[23] Despite this, NCCN does strongly advocate for broader molecular profiling for identifying rare driver mutations.[23] In contrast, the CAP/IASLC/AMP guidelines stated that multiplexed genetic sequencing panels (such NGS) are preferred over multiple single-gene tests to identify other treatment options after testing for EGFR, ALK, and ROS1.[21] This preference is based on the limited amount of tissue obtained from patients and the ever-increasing list of molecular targets which may provide therapeutic guidance.

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The limitation of tissue is another key area of concern given that the initial assessment of patients diagnosed with NSCLC is most commonly performed on fine needle aspirates (FNAs) or core needle biopsies (CNBs). The size of such samples results in significant limitations on the testing methodologies available and will continue to do so, in response to continued efforts to have minimally invasive procedures to reduce patient discomfort. Therefore, the use of NGS, and the possibility of identifying novel mutations in a single test, may become an appealing alternative. Indeed, even in the community-based practices, NGS testing appears to be gaining traction, increasing by approximately 15% in five years.[24] The adoption of NGS technology in the clinical laboratory is increasing despite the complexity of the methodology; NGS requires multiple steps, often over multiple days, followed by a comprehensive analysis of collected data. Moreover, NGS uses multiplexed, high-throughput parallel sequencing, relying on bioinformatics algorithms (which can be experimental) to identify genetic anomalies by comparing them to reference sequences and known genetic variants housed in public or proprietary databases. And within the realm of tumor profiling, the constructed libraries used as the source for amplification and sequencing need to be further enriched to focus on the desired genetic targets associated with tumors. This is often achieved by the additional step of either hybrid-capture with specific oligonucleotides designed to capture the targets of interest or with amplicon PCR where the targets are amplified, after which the focused library is amplified and sequenced.

TESTING FOR ALK WITH NGS

In the case of ALK, FISH technology was the first approved CDx for ALK rearrangements; however, technological advancements have led to competition among testing platforms, resulting in validated IHC assays being included in the most recent guidelines.[1,21,25] Both FISH and IHC technology have been at the forefront of ALK testing, but recently NGS has been expanding options for ALK testing as well, providing initial tumor profiling and therapy management. It is, therefore, necessary to assess whether NGS is a viable alternative to identify ALK gene rearrangements in NSCLC samples. The genetic abnormalities identified using NGS can provide information on targetable mutations, advance clinical research, and direct patients with limited therapeutic options into clinical trials.[26,27] However, there are drawbacks to the extensive information obtained from NGS testing. The technology is often criticized for providing clinicians results beyond those that are actionable by including results with undetermined clinical significance.[28] And when it comes to the specific identification of ALK rearrangements, NGS assays can be limiting. For instance, although the FDA-approved MSK-IMPACT assay provides information on numerous genes, it can only identify several known EML4-ALK translocations.[29]

The use of targeted NGS for ALK rearrangements requires prior knowledge of the potential fusion partners. The current consensus is that ALK has more than 20; therefore, it is unlikely that NGS would identify novel or rare ALK rearrangements.[30] Another commercially available IVD/CE marked test, Oncomine Dx (Life Technologies), does not currently claim to identify ALK rearrangements in the US; however, it is often modified and further validated in the laboratory purchasing it. Currently, there is one other FDA approved test, FoundationOne CDx (Foundation Medicine), which provides information for multiple tumor types and genetic targets. Although it should be noted that this test was validated with a 92% accuracy in identifying positive cases when compared to the FDA approved FISH and IHC tests, this rate fell to 85.9% accuracy when compared to only the FISH test.[31] (See Table 3). In addition to these NGS tests, there are numerous others available for research use only and validated in laboratories, with variations in the sensitivity and specificity of the assays, the percent of tumor tissue needed, the limit of detection, and the success rate.[32,33] When compared to FISH and IHC, the sensitivity of NGS assays has been documented to be as low as 80%, with the expected limitations of the less common rearrangements.[32,34]

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TABLE 3: NGS ACCURACY IDENTIFYING ALK REARRANGEMENTS COMPARED TO FISH

NGS ASSAY	POSITIVE % AGREEMENT	NEGATIVE % AGREEMENT
FoundationOne CDx[31]	85.9% (79/92)	96.4% (80/83)
Oncomine Fusion Transcript Kit[33]	91.7% (11/12)	99.3% (137/138)
RNA Fusion Lung Cancer Research Panel[32]	82.9% (29/35)	94.7% (18/19)

Another factor to consider is that with NGS, the requirements for a quality sample of extracted DNA or RNA are much greater. When considering the limitations of the sample type obtained from NSCLC patients, this becomes problematic. Whereas a test like ALK FISH would require one H&E stained slide and one FISH slide, the requirements for NGS may require up to 10 slides, which can be challenging with the smaller sample sizes that are collected with FNA and CNB.[35] (See Figure 2). Moreover, NGS assays have a lower success rate (based on availability and quality of sequencing data), often due to processing issues.[36–38] Therefore, careful processing of NGS samples is essential, since processing factors such as storage conditions and time can interfere with the integrity of the sample.[39] As a result, using NGS methodology as the primary test increases the risk of exhausting the sample prior to obtaining successful and actionable results.

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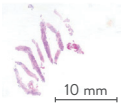
FIGURE 2: SLIDE REQUIREMENTS BY TYPE OF SAMPLE COLLECTED AND TECHNOLOGY

TYPE OF SAMPLE COLLECTED FOR DIAGNOSIS AND MOLECULAR PROFILING
(dependent on location and accessibility)

LEAST INVASIVE

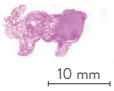
FNA—Fine Needle Aspirates
Sample Size: Tiny

CNB—Core Needle Biopsy
Sample Size: Small



MOST INVASIVE

Resected Tissue Biopsy
Sample Size: Medium/Large



NUMBER OF SLIDES needed per technology

IHC	FISH	NGS
3 Slides 1–H&E 1–Assay 1–Assay Control	**2 Slides** 1–H&E 1–Assay	**≥10 Slides**
3 Slides 1–H&E 1–Assay 1–Assay Control	**2 Slides** 1–H&E 1–Assay	**6–10 Slides**
3 Slides 1–H&E 1–Assay 1–Assay Control	**2 Slides** 1–H&E 1–Assay	**1–5 Slides**

H&E: hematoxylin and eosin stain

Another critical factor to consider for any ALK testing method is the guideline assertion that results must be available within two weeks or less for physicians to implement the best course of treatments. A 2017 assessment of real world clinical practice showed that results were delayed by more than 4 weeks for 34% of patients tested for ALK rearrangements, with the possibility of only 42% of those patients receiving appropriate therapy.[40] Although the technology is continuing to advance and reduce the time to result, in many cases, the time to obtain NGS results may take longer than two weeks, since an extensive computer analysis and pathologist interpretation occurs after the complex processing to obtain raw data. Therefore, for laboratories performing NGS testing in house and those forwarding the samples to NGS testing centers, meeting the time-to-result recommendation is a challenge with the currently available NGS assays, potentially delaying the placement of patients into proper treatment protocols.

BEST COURSE OF ACTION

When assessing the advantages and disadvantages of the different technologies currently available for ALK testing, one of the most important considerations is how they influence and affect patient health. As the number of actionable mutations expands, the amount of tissue collected does not, and the need for sample conservation is increased, thus limiting the number of biomarker tests performed on a sample. Pathologists are now increasingly required to exercise caution in specimen processing, conservation of tissue, and biomarker test selection to ensure that actionable information is provided.[17,19,41] Following the recommendations of testing actionable targets such as ALK first, laboratories need to consider a variety of factors: turnaround time, test volume, the total cost for test execution, reimbursement, effects of workflow, and detection rate of ALK positivity.[42]

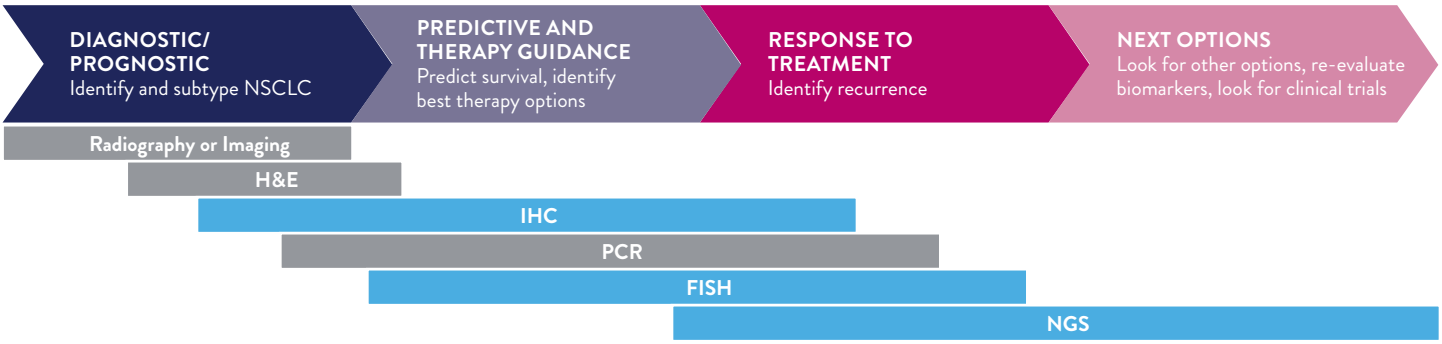
Most ALK testing algorithms used today involve either FISH or IHC testing, with many including both tests at some stage.[15,17] Although IHC testing has come close to matching the sensitivity and specificity of FISH, the potential need to follow-up or reflex with FISH testing for negative or challenging cases may negate the perceived advantages of a lower cost and need for less technical expertise. Likewise, the current state of NGS technology has not yet reached the level of sensitivity and specificity of FISH or IHC for the detection of ALK rearrangements, and NGS can greatly extend the time to result and consequently patient treatment for laboratories processing samples in-house and those sending samples to external sites. Furthermore, the risks of invalidated outcomes due to the difficulty in isolating optimal testing material for NGS make it less than ideal for testing for ALK rearrangements. Although NGS provides an array of genomic information, most of the information is not clinically actionable—there are no therapies that target most of the specific mutations identified, and the prognostic information may be equally unclear.

It is important to weigh these considerations, along with the cost, the longer turnaround time, and the lower success rate of testing associated with NGS when selecting a method for ALK testing. With the current limitations of NGS testing, FISH and IHC have a clear advantage as preferred methods for ALK gene rearrangement testing.

It is important to weigh these considerations, along with the cost, the longer turnaround time, and the lower success rate of testing associated with NGS when selecting a method for ALK testing. With the current limitations of NGS testing, FISH and IHC have a clear advantage as preferred methods for ALK gene rearrangement testing.

The overall path of testing for an NSCLC patient can encompass all the previously mentioned technologies. Each type of testing (IHC, FISH, and NGS) has a role in the patient care life cycle (See Figure 3 on page 11) beginning with the initial diagnosis of NSCLC, followed by a prognostic and predictive evaluation and optimal treatment assessment, then later in monitoring the response to treatment, and finally, the search for other options for refractory or relapsed patients.

FIGURE 3: PATIENT CARE LIFE CYCLE AND DIAGNOSTIC TESTING



CONCLUSIONS

Genomic testing standards for NSCLC are expanding and evolving rapidly. Laboratories and clinicians must be cautious about how new technologies, diagnostics approaches, and therapies are incorporated into practice to ensure the quality and consistency of testing methods, providing clinicians with actionable results. Today, FISH is still considered the gold standard for ALK testing because of its sensitivity and specificity, relative cost, and rapid turnaround time. For front-line testing of actionable genetic alterations such as ALK (or ROS1) rearrangements, FISH is appropriate either as a standalone test or in combination with IHC, while NGS is optimal if more tissue is available or if the initial molecular profiling deems it necessary. As NGS methodology continues to improve and become clinically validated, it will reach a point where it will be able to replace FISH and IHC for testing in certain scenarios. However, today, FISH enables laboratories to provide prompt, accurate molecular testing results to physicians, facilitating treatment decisions that allow patients to achieve the best possible outcomes in a disease that was once considered to have a very poor prognosis.

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