Crizotinib and Testing for ALK

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Abstract
Crizotinib was recently approved by the US FDA for the treatment of advanced non–small cell lung cancer (NSCLC) harboring the ALK (anaplastic lymphoma kinase) gene rearrangement. To ensure identification of patients most likely to benefit, the FDA approved crizotinib concurrently with a companion diagnostic test—the Vysis ALK Break Apart FISH Probe Kit. This kit was used in 1 of the 2 pivotal trials leading to the FDA approval of crizotinib and has become the gold standard for detecting ALK rearrangement in NSCLC. Although ALK FISH is clinically validated, the assay can be technically challenging and costly. Therefore, other diagnostic modalities are being explored, including immunohistochemistry (IHC) and reverse transcriptase–polymerase chain reaction. This article provides an overview of the diagnostic assays available for detecting ALK rearrangement. Each assay, including ALK FISH, has its strengths and weaknesses. Recent work with commercially available ALK antibodies suggests that IHC-based tests may represent a reliable and cost-effective screening strategy; however, large multicenter studies comparing IHC with FISH are needed to validate ALK IHC. While ALK FISH remains the current standard for diagnosing ALK positivity, large-scale screening of patients with newly diagnosed advanced NSCLC, as recommended by NCCN, may require development and validation of alternative screening strategies, such as combination IHC and FISH. (JNCCN 2011;9:1335–1341)

Anaplastic lymphoma kinase (ALK) was first identified as a potential drug target in non–small cell lung cancer (NSCLC) 4 years ago when it was discovered as a fusion kinase in a small percentage of Japanese patients with NSCLC.\textsuperscript{1} This discovery fueled the development of a diagnostic assay for ALK rearrangement,\textsuperscript{2} which in turn enabled the identification and recruitment of patients with ALK-positive NSCLC into a phase I clinical trial of crizotinib, a dual MET/ALK tyrosine kinase inhibitor.\textsuperscript{3} In this and a subsequent phase II trial, crizotinib was shown to be highly active, with an objective response rate of approximately 50% to 60%.\textsuperscript{4,5} Based on its efficacy and safety, crizotinib was recently granted accelerated approval by the FDA for the treatment of patients with advanced ALK-positive NSCLC. As with the BRAF inhibitor vemurafenib for BRAF mutant melanoma,\textsuperscript{6} crizotinib was approved with a companion diagnostic test. This test—the Vysis ALK Break Apart FISH Probe Kit (Abbott Molecular)—was used to identify patients with ALK-positive disease in the phase II study of crizotinib, and is now required by the FDA to offer patients treatment with crizotinib.
Rearrangements of the ALK gene are relatively uncommon events in NSCLC, but they have profound therapeutic implications for patients identified as ALK-positive. The diagnostic test for ALK rearrangement must therefore be accurate, accessible, and affordable. The FDA label for crizotinib has firmly established fluorescence in situ hybridization (FISH) as the current gold standard for detecting ALK rearrangements in the United States. However, several other methods for establishing ALK positivity have been studied, including immunohistochemistry (IHC) using different ALK-specific antibodies and reverse transcriptase–polymerase chain reaction (RT-PCR). This review discusses each of the available methods, with a focus on the potential utility of non-FISH methods in selecting patients for treatment with crizotinib. How current methods for detecting ALK could impact screening algorithms for NSCLC patients is also discussed.

**ALK FISH: The Current Gold Standard**

The break apart FISH assay for detecting ALK rearrangements has served as the standard test for establishing ALK positivity in clinical trials of crizotinib. The commercial break apart probes consist of 2 differently colored probes (red and green) that flank the highly conserved translocation breakpoint within ALK. In cells with non-rearranged ALK, the overlapping red and green probes result in a fused or yellow signal; in the setting of an ALK rearrangement, these probes are spatially separated, resulting in splitting of the red and green signals (Figure 1A). In practice, this split pattern is seen in approximately 70% of ALK-positive cases; in the remaining approximately 30%, FISH shows a different pattern—an isolated 3′ or red signal (Figure 1B)7,8 (A. Iafrate, personal communication). FISH-positive cases are defined as those with more than 15% split or isolated red signals among 50 tumor nuclei scored; this threshold was established based on the observation that 15% split or isolated red signals is 2 standard deviations above the average number of split or isolated red signals detected in formalin-fixed, paraffin-embedded (FFPE) non-tumor control tissue.

ALK FISH is widely regarded as clinically validated because this methodology was the diagnostic test used to identify ALK-positive patients in the phase I and II trials of crizotinib, the results of which led to FDA approval. In addition, ALK FISH testing is also mandated in the ongoing confirmatory phase III trials of crizotinib (ClinicalTrials.gov identifiers: NCT00932893 and NCT01154140). The major advantage of ALK FISH is that this assay is capable of detecting any rearrangement involving ALK, including potentially rare, uncharacterized ALK rearrangements. From a technical standpoint, ALK FISH is performed on FFPE tissue, which represents the most common method for processing and storing tumor specimens. Even one unstained slide cut from an

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**Figure 1** Break-apart anaplastic lymphoma kinase (ALK) fluorescence in situ hybridization (FISH) assay. (A) The most common pattern associated with ALK rearrangement is splitting of the red and green signals (arrows). (B) Isolated red signals (arrowheads) are also associated with ALK rearrangement and are found in approximately 30% of positive cases. From Shaw AT, Forcione DG, Digumarthy SR, Iafrate AJ. Case 21-2011: a 31-year-old man with ALK-positive adenocarcinoma of the lung. N Engl J Med 2011;365:162; with permission. Copyright © 2011 Massachusetts Medical Society.
FFPE block may be sufficient for ALK FISH analysis. The FDA-approved ALK FISH kit was optimized for screening FFPE human NSCLC tissue, specifically core and resection specimens, but ALK FISH testing has also been performed on cytology specimens using a modified protocol, with cell blocks preferred over smears (A. Iafrate, personal communication). Thus, most patients with NSCLC will have tumor tissue appropriate for ALK FISH testing.

Using ALK FISH for the routine detection of ALK rearrangement in NSCLC also has several important disadvantages. First, FISH generally requires specialized technical resources and expertise and is therefore not available in all pathology laboratories. In particular, due to (1) the intrachromosomal deletion and inversion event that underlies most ALK rearrangements in NSCLC and (2) variability in the precise fusion of ALK with partner proteins such as EML4, the readout of ALK FISH can be challenging. On occasion, splitting of the red and green signals can be extremely subtle, leading to false-negative results. In addition, several NSCLC cases have been referred to the authors’ institution as a result of discrepant ALK FISH interpretation between 2 specialized laboratories.

Second, because FISH requires fluorescence microscopy, the tissue architecture and morphology of individual tumor and non-tumor cells cannot be evaluated. A related method, chromogenic in situ hybridization (CISH), which uses digoxigenin- and 2,4-dinitrophenyl-labelled ALK probes and bright field microscopy, may overcome this specific problem. However, this method is not widely available, and, as with FISH, results can be difficult to interpret because of atypical or subtle splitting patterns. Finally, although the cost of ALK FISH testing is still in flux, it may be substantial, with some estimates as high as $1500 per patient. This cost presents a significant barrier to large-scale screening of patients with advanced NSCLC and highlights the need to develop and validate more cost-effective methods for detecting ALK rearrangements.

**ALK IHC: The Next Frontier of Molecular Diagnostics**

Although ALK FISH currently represents the gold standard for diagnosing patients with ALK-positive NSCLC, ALK IHC may soon become the diagnostic test of choice. In general, IHC is a rapid and affordable method preferred by pathologists for routine screening and diagnosis. Similar to ALK FISH, in theory, IHC requires only one unstained slide cut from a FFPE block, provided it includes at least a few clusters of preserved tumor cells. IHC can be performed successfully on a variety of different tumor specimens and on fine-needle aspiration cell blocks. The major challenge to using ALK IHC is the often low-level expression of ALK fusion proteins in ALK-rearranged NSCLC, which has necessitated the development of more sensitive IHC-based methods. Other disadvantages of IHC include lack of quantitation, interobserver variability, and sensitivity to tissue fixation. The third issue could lead to false-negative results and decreased sensitivity in detecting ALK rearrangement.

Early studies with DAKO clone ALK1, a mouse monoclonal ALK antibody used commonly in pathology laboratories worldwide, showed that this antibody was specific but not sensitive for detecting ALK expression secondary to ALK rearrangement in NSCLC. In this study, IHC using the ALK1 antibody identified only 4 of 10 cases identified as positive for ALK rearrangement using FISH. Increasing the antibody concentration from a normal 1:50 dilution (used in detecting ALK expression in NPM-ALK fusion-positive anaplastic large cell lymphoma) to a 1:2 dilution increased the sensitivity to 60%. Diluting the primary antibody and secondarily amplifying the signal with a tyramide-biotin–based protocol boosted the sensitivity to only 80%.

To enhance the detection of ALK protein expression, Takeuchi et al. developed an intercalated antibody-enhanced polymer (iAEP) method. In their study with 11 EML4-ALK–expressing specimens and 10 normal controls, IHC using the ALK1 antibody with iAEP had 100% sensitivity and 100% specificity, though a low-level of background staining was noted with this approach. More recently, Yi et al. tested the ALK1 antibody in conjunction with DAKO’s ADVANCE detection system in 101 NSCLC samples, all of which had also been screened using ALK FISH. When scores of 2+ and 3+ were defined as IHC-positive (and 0 and 1+ as IHC-negative), the sensitivity and specificity of this IHC method was 90% and 98%, respectively, with moderate to substantial interobserver and intraobserver concordance.

Several other ALK antibodies have also been...
One of the most promising antibodies for the detection of ALK rearrangement in NSCLC is clone D5F3, a rabbit monoclonal ALK antibody developed by Cell Signaling Technology. In a recent study of 153 NSCLC specimens, 22 of which were confirmed as ALK-positive using ALK FISH, the sensitivity and specificity of D5F3 at 1:100 dilution were remarkably high—100% and 99%, respectively—with excellent interobserver agreement among 3 pathologists (κ statistic, 0.94). In contrast, using the ALK1 antibody, the sensitivity of IHC was only 67%, though the specificity remained high at 97%. Although IHC using the D5F3 antibody showed moderate to strong staining of ALK-rearranged cases, IHC with the ALK1 antibody (even at high concentration) resulted in only weak staining of the same cases (Figure 2A), with considerable overlap in staining intensity between ALK-positive and ALK-negative NSCLC tumors. The findings with D5F3 require validation in larger NSCLC series, and such studies are on hold until the antibody becomes commercially available. However, the near-perfect concordance between ALK IHC and ALK FISH shown in this study suggests that the D5F3 antibody could serve as the basis for an ALK screening test and eventually obviate routine ALK FISH.

A third ALK antibody that has been tested in NSCLC and is commercially available is the 5A4 monoclonal antibody from Novocastra. As with the ALK1 study by Yi et al., this study also used a 4-tiered scoring system in assessing immunoreactivity. IHC was performed using 5A4 at a dilution of 1:30 with a Ventana automated immunostainer. The sensitivity and specificity of IHC were shown to be 100% and 96%, respectively. All tumor specimens with ALK IHC scores of 3+ were ALK FISH–positive, those with IHC scores of 0 or 1+ were ALK FISH–negative, and those with a score of 2+ were ALK FISH–variable. Based on these results, the 5A4 antibody could be used to screen for ALK rearrangement, with tumors that show 2+ or 3+ IHC positivity confirmed (or not confirmed) by ALK FISH. At Massachusetts General Hospital, the authors recently implemented IHC using 5A4 with Leica automation and no enhancing system. The ALK FISH–positive cases examined to date have shown moderate to strong IHC staining (Figure 2B). For 5A4 as well as ALK1, if the proposed IHC scoring systems are found to be accurate in detecting ALK rearrangements in larger-scale concordance studies, IHC could become the primary screening modality for ALK-positive NSCLC, with only IHC 2+ and 3+ cases requiring confirmation with the FDA-approved ALK FISH test.

**RT-PCR: An Alternative Diagnostic Assay**

RT-PCR was used to identify ALK rearrangements in many early retrospective series of ALK-positive NSCLC. It is a highly sensitive technique for detection and quantification of RNA for EML4-ALK that also allows for sequencing of the PCR product.
product to identify the specific EML4-ALK variant expressed. The assay has traditionally required extraction of RNA from fresh-frozen tissue samples, which are not routinely obtained in the diagnostic workup. However, the assay may be optimized to allow testing of archived FFPE tissue\textsuperscript{10,20–22} and small clinical samples such as those obtained from endobronchial ultrasound transbronchial needle aspirate.\textsuperscript{23,24} Individual primer pairs must be designed to amplify particular ALK fusions, but assays may be multiplexed to detect many if not all of the known EML4-ALK rearrangements.\textsuperscript{10,20,22}

As an example, Li et al.\textsuperscript{21} recently reported a large-scale analysis of 1889 archival FFPE specimens using a multiplexed RT-PCR assay designed to detect 9 EML4-ALK variants. They identified 75 NSCLC cases (4%) as positive for EML4-ALK expression. Positive cases had several of the clinicopathologic features associated with ALK rearrangement, including younger age and adenocarcinoma histology. This multiplexed RT-PCR assay is the basis for the commercial diagnostic assay for ALK rearrangement offered by Response Genetics.

The major disadvantage of RT-PCR is that minimal data comparing the results of RT-PCR screening with either ALK FISH or ALK IHC are available. In the study by Li et al.,\textsuperscript{22} concordance with a second method of ALK testing was not reported. A second disadvantage of RT-PCR is that this methodology will not identify novel rearrangements involving previously uncharacterized fusion partners. This leads to potential false-negative results. Conversely, another important drawback to RT-PCR is the possibility of contamination that might impair specificity and lead to false-positive results. In tissues with borderline preservation, RT-PCR will more likely fail compared with FISH or IHC, because RNA is typically more sensitive to degradation than DNA or protein. Given these limitations and the absence of concordance data, positive results by RT-PCR should be confirmed using other diagnostic methods.

**Implications for ALK Testing: Who and How?**

In the United States, crizotinib is an approved therapy for patients with advanced ALK-positive NSCLC, as determined by the FDA-approved test for ALK rearrangement. Currently, the only FDA-approved test is the Vysis ALK Break Apart FISH Probe Kit. The updated NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines) for NSCLC recommend upfront molecular testing of all patients with nonsquamous histology for both EGFR mutation and ALK rearrangement, because both genetic abnormalities are immediately actionable (to view the most recent version of these guidelines, visit the NCCN Web site at www.NCCN.org). Although ALK rearrangement is associated with certain clinical features, such as younger age and never-smoking history, there are by no means absolute associations, and both older patients and patients with a smoking history have been shown to be ALK-positive as well as responsive to crizotinib.\textsuperscript{3}

Given the potential price of the approved ALK FISH test, however, the feasibility of screening all patients with nonsquamous NSCLC is uncertain. One strategy to help address this is to perform sequential EGFR mutation testing followed by ALK FISH in newly diagnosed patients, because EGFR and ALK are generally mutually exclusive. Sequential testing may help offset a small portion of the cost but will lead to a delay in ALK testing and hence a delay in treatment with first-line crizotinib. In previously treated patients who are not known to harbor mutations in EGFR or other oncogenic drivers such as KRAS, testing with ALK FISH should be offered.

Restricting ALK FISH testing to patients with nonsquamous histology in the NCCN Guidelines for NSCLC is based on the observation that among the 255 ALK-positive patients included in the FDA submission, only 1 (0.4%) had squamous cell histology and 3 (1.2%) had mixed adenosquamous histology. At least 2 single-institution series have reported an exceedingly low rate of ALK positivity among patients with squamous cell carcinoma (e.g., 0 of 163 patients\textsuperscript{7} and 1 of 200 patients\textsuperscript{25}). Although routine ALK FISH testing of patients with squamous cell carcinoma histology is not supported by the available data, the FDA label for crizotinib does not specify nonsquamous histology, and it would be reasonable to screen select patients with squamous cell lung carcinomas on a case-by-case basis. Several studies have identified a handful of patients with ALK-positive adenosquamous or squamous cell carcinoma.\textsuperscript{1–3,26} These patients tend to be never- or light-smokers, which is distinctly uncommon with squamous cell histology, and have been noted to respond to crizo-
Conclusions

The accurate diagnosis of ALK positivity is critically important because it identifies patients who are eligible for treatment with crizotinib, a targeted therapy that almost certainly impacts the natural history of ALK-positive NSCLC. As described in the NCCN Guidelines for NSCLC, the current standard method for detecting ALK rearrangement is ALK FISH. Although every patient with advanced NSCLC should be offered ALK testing so as not to deprive any ALK-positive patient of treatment with crizotinib, at the present time, the cost of the standard ALK FISH test is prohibitive to large-scale screening efforts. Assuming the prevalence of ALK rearrangement is approximately 5% in NSCLC and the cost of ALK FISH testing is $1000 per patient, the cost to identify one ALK-positive patient would be $20,000. These types of calculations are driving the development of alternative screening strategies for ALK rearrangement that are as accurate as ALK FISH but more cost-effective.

To date, IHC represents the most promising alternative to FISH in terms of both reliability and cost. Screening IHC algorithms using commercially available ALK antibodies such as ALK1 and 5A4 have been proposed based on studies showing high concordance with ALK FISH. Because IHC is a routine and affordable technique used in regular pathology laboratories, an IHC-based ALK test would improve the accessibility and cost-effectiveness of ALK testing and facilitate large-scale screening efforts. However, before implementing large-scale screening using ALK IHC, multicenter, prospective studies are required to validate concordance between IHC and FISH. Academic centers with experience in ALK IHC may already be using one of the proposed ALK IHC algorithms, but until ALK IHC is clinically validated and FDA-approved, patients identified as ALK-positive by IHC will still require confirmatory ALK FISH testing to be eligible for crizotinib. In the near future, IHC or a combination of IHC and FISH may be routinely used to enable the largest number of patients to be screened for ALK rearrangement. Ultimately, however, the best diagnostic strategy for ALK-positive NSCLC awaits further study and validation.

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