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Diagnostic Assays for Identification of Anaplastic Lymphoma Kinase–Positive Non–Small Cell Lung Cancer

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Abstract

In series dominated by adenocarcinoma histology, approximately 5% of non–small cell lung cancers (NSCLCs) harbor an anaplastic lymphoma kinase (*ALK*) gene rearrangement. Crizotinib, a tyrosine kinase inhibitor with significant activity against *ALK*, has demonstrated high response rates and prolonged progression-free survival in *ALK*-positive patients enrolled in phase 1/2 clinical trials. In 2011, crizotinib received accelerated approval from the US Food and Drug Administration (FDA) for the treatment of proven *ALK*-positive NSCLC using an FDA-approved diagnostic test. Currently, only break-apart fluorescence in situ hybridization testing is FDA approved as a companion diagnostic for crizotinib; however, many other assays are available or in development. In the current review, the authors summarize the diagnostic tests available, or likely to become available, that could be used to identify patients with *ALK*-positive NSCLC, highlighting the pros and cons of each.

Keywords

crizotinib; non-small cell lung cancer; anaplastic lymphoma kinase (*ALK*) gene rearrangements; fluorescence in situ hybridization

INTRODUCTION

Patients with non–small cell lung cancer (NSCLC) who harbor anaplastic lymphoma kinase (*ALK*) gene rearrangements can derive significant clinical benefit from crizotinib (Xalkori; Pfizer, La Jolla, Calif), a small molecule inhibitor of the *ALK* tyrosine kinase.¹ In August 2011, crizotinib received accelerated approval from the US Food and Drug Administration (FDA) for the treatment of proven *ALK*-positive NSCLC using an FDA-approved diagnostic test.² To date, only fluorescence in situ hybridization (FISH) using the Vysis break-apart probe kit (Abbott Molecular, Des Plaines, Ill) is FDA approved for this

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CONFLICT OF INTEREST DISCLOSURES

Drs. Weickhardt, Doebele and Camidge have received speaking honoraria from Pfizer. Dr. Garcia has received a speaking honorarium from Abbott Molecular.

indication. However other tests exist, and several of those are, or may become, commercially available and may seek regulatory approval in the future.

ALK Biology

ALK is a transmembrane receptor tyrosine kinase involved in development that is largely silenced in most adult tissues.³ Oncogenic activation of ALK can occur through several different mechanisms.⁴ Although primary activating mutations have been described in other cancers, ALK activation in NSCLC involves gene rearrangements that place 1 of several different 5' fusion partners and their associated promoters upstream of the region encoding the 3' kinase domain of *ALK*.⁵⁻⁷ The most common *ALK* rearrangement in NSCLC is a paracentric inversion on the short arm of chromosome 2 juxtaposing the 5' end of the echinoderm microtubule associated protein-like 4 (*EML4*) gene with the 3' end of the *ALK* gene.⁸ Rare reports of constitutive activation of the ALK tyrosine kinase resulting from fusion with genes other than *EML4* in NSCLC, including transforming growth factor (*TFG*), kinesin family member 5B (*KIF5B*), kinesin light chain 1 (*KLC1*), and protein tyrosine phosphatase nonreceptor type 3 (*PTPN3*), also have been described.⁹⁻¹² In the *EML4-ALK* fusions, it is known that the 5' *EML4* partner can be variably truncated, creating different *EML4-ALK* variants, the biologic significance of which is under investigation.^{7,13-15}

ALK Testing in Non-small Cell Lung Cancer

Although ALK activation in NSCLC was described only in 2007, since the middle 1990s, multiple different methods for detecting ALK activation have been explored in the lymphoma field, several of which have been applied to NSCLC.¹⁶⁻¹⁸ In addition, new techniques have been developed since the discovery of ALK in NSCLC. The pros and cons of these different techniques are described in detail below and in Table 1. A schematic of the major techniques is provided in Figure 1.¹⁹⁻²¹

Fluorescence in situ hybridization—Fluorescence in situ hybridization (FISH) testing involves using fluorescently labeled DNA probes to bind to and localize specific genomic regions in the tumor nuclei on tissue sections. The Vysis *ALK* break-apart FISH test (Abbott Molecular) was used as the diagnostic test in all of the initial and ongoing registration clinical trials of crizotinib.^{1,22} *ALK* break-apart FISH sets also have been used in conjunction with separate break-apart FISH probe sets focusing on the partner genes (eg *EML4* break-apart probe sets).^{12,23} Fusion probe sets, which use fluorescent probes to identify the specific translocation partner for FISH, also exist, but none of these alternative FISH techniques been used prospectively in any ALK inhibitor trials to date.^{24,25}

Break-apart *ALK* probe set—The 5' and 3' fluorescent probes in the Vysis break-apart assay bind to areas upstream and downstream, respectively, of the common rearrangement breakpoint in exon 20 of the *ALK* gene. In the color pairing used in the commercial assay, the 5' probe is labeled with green fluorophores, and the 3' probe is labeled with orange fluorophores (but the signal generated is usually detected with the interference filter set in the red wave length range and, thus, is observed as red). In the normal *ALK* gene state, the red and green signals are sufficiently closely apposed that, together, they are referred to as a fused signal, sometimes registering as a composite yellow signal. Within each cell, the different component signals (red, green, or fused) form different positive or negative patterns. When rearrangement occurs, the red and green signals separate farther apart than in the native state. In the classic “split” positive pattern, isolated red and green signals separated by at least 2 signal diameters are observed, even a single example of which is sufficient to designate a cell as positive. In addition, if an isolated red (3') signal occurs without a coexistent green signal being present to identify a split pattern, then such “single red” patterns also are considered positive.^{1,26} A single red pattern is presumed to indicate

that a rearrangement has occurred with either loss of the 5' probe binding site completely or within the plane of section. One positive pattern (split or single red) usually prevails in tumors, suggesting that true 5' loss may be occurring, although mixed positive patterns can be observed.²⁶ In contrast, fused and single green (5') patterns (given that the latter may involve loss of a binding site located in the kinase domain itself) are considered negative in relation to predicting benefit from ALK inhibitor intervention.²⁶ It is noteworthy that, because not every copy of ALK in a cancer cell is rearranged, both fused and rearranged signals coexist in the same cell (ie, in a positive cell, at least 1 fused signal and 1 isolated red signal, either with or without an isolated green signal per cell, are likely to be observed). The different patterns are illustrated in Figure 1b.

Although the detection of specific patterns determines whether an individual cell is counted as a positive, it is the overall proportion of positive cells across multiple cells in the same tumor that determines whether the tumor itself is designated ALK-positive or ALK-negative. Copy number gain of both native and rearranged *ALK* signals has been noted in NSCLC. Although copy number gain of rearranged signals has been associated with acquired resistance to crizotinib, the mean copy number per cell of either type of signal is not currently considered in the determination of initial ALK positivity.²⁷⁻²⁹ In vitro responses to very high concentrations of crizotinib (median, 1750 nM) have been noted in cell lines manifesting native copy number gain.³⁰ However, at more physiologically achievable doses (<250 nM), there appears to be no association with native copy number and responsiveness to crizotinib.³¹ In addition, in most cases of native copy number gain, the pattern of gain is diffuse within tumor cells, consistent with it reflecting general chromosomal aneusomy rather than a specific selection for this region. Abundant focal amplification of native signals does occur, but it is very rare (0.8% of ALK negative cases) and may be worthy of more detailed exploration in the future as an "atypical negative" (see below).³¹

In the clinical trials of crizotinib, a tumor specimen was defined as positive for an *ALK* rearrangement if >15% of scored tumor nuclei demonstrated a positive pattern.^{1,26} Within the FDA-approved companion diagnostic, this percentage currently is listed as 15%. This cutoff point was chosen because it appeared to sit within a natural gap in the continuum of "percentage cells positive" that allows distinguishing true-positive tumors from the technical background noise of the assay.^{26,32,33} More recently, as larger numbers of samples have been analyzed, small proportions of negative samples appear to closely approach the >15% cutoff point for determining tumor positivity.³¹ To reliably determine whether any of these may represent false-negatives, either reinterrogation of the samples with alternative diagnostic techniques and/or an assessment of their response to crizotinib would be required. It is noteworthy that several retrospective and prospective studies evaluating different diagnostic techniques and prospective studies evaluating the response to crizotinib of borderline and atypical negative cases (such as single green signals or native copy number gain) are planned under both commercial and cooperative oncology group guidance.

Because positive cell counts in positive tumors usually are far less than 100%, does this reflect clonal heterogeneity within the tumor, with *ALK* rearrangement considered either a late event in tumorigenesis or an expendable event in some situations, or does it reflect false cellular negative results generated within the assay?²⁵ When a series of contiguous high-power fields of ALK-positive tumors were evaluated, and each individual field was assessed for whether it did or did not meet positive tumor criteria, no evidence of positivity as a focal event could be observed.²⁶ There appears to be no correlation between the percentage positive cells and the maximal percentage shrinkage according to Response Evaluation Criteria in Solid Tumors (RECIST) in ALK-positive tumors treated with crizotinib (correlation coefficient [r] = 0.192; P = .3).³⁴ In addition, there is a strong linear correlation

between the percentage positive cells and the copy number of isolated red signals (contributing to both of the accepted positive patterns; ie, split and single red patterns; $r = 0.743$; $P = .0001$).³⁴ This finding is consistent with multiple copies of the relevant signal increasing the chances of detecting a positive pattern in any given cell.³⁴ Overall, these data suggest that the majority of negative cells in ALK-positive tumors represent false cellular negatives rather than true biologic negatives.

Given the potential for both false cellular positives and negatives with the break-apart FISH assay, analyses of very small numbers of cells could be misleading.²⁶ The FDA-approved assay requires counting a minimum of 50 tumor cell nuclei for a first reader with a requirement for counting an additional 50 cells by a second reader in cases with >10% but <50% positive tumor cell nuclei.

In terms of the quantity and quality of tissue required to conduct the ALK FISH assay, even very small samples of formalin-fixed, paraffin-embedded (FFPE) tissue or a single unstained section on a slide may be adequate for analysis. Bone biopsies usually are considered unsatisfactory for FISH analysis because of the effect of decalcification on the quality of the genomic DNA.³⁵ This may sometimes be avoided by the use of ethylene diamine tetra-acetic acid-based decalcification agents.^{32,33} Alternatively, a bone aspirate performed after the bone biopsy needle is inserted can be used to prepare a cell pellet suitable for further processing without requiring decalcification treatment.

The Implications of US Food and Drug Administration Approval of the Vysis Break-Apart Probe Set

Within the initial phase 1 study of crizotinib, the ALK break-apart FISH assay was used by means of purchased reagents at individual investigators' sites. Within subsequent studies, a kit version of the same assay was used, and a central vendor was used to form the basis for FDA approval of the Vysis kit.

Generally, FDA approval reduces the requirement for individual laboratories to perform extensive clinical and technologic validation for the assay. Specifically, a laboratory can conduct a verification (instead of a validation) to confirm that the assay performs as intended in the course of routine operations. This also reduces the upfront costs to establish an assay in a particular laboratory. However, to remain "on-label," all elements of the assay must be performed exactly according to FDA-approved instructions. This includes identification of specific pieces of equipment by brand and model number and branded reagents. For laboratories that already offer similar testing, the procurement of a specific piece of instrumentation exclusively for the assay and the use of specifically branded reagents when a laboratory already has established procedures for similar assays may be problematic. Not surprisingly, FDA-approved assays often cost more than their non-FDA-approved counterparts. In addition, once assays become FDA approved, the non-FDA-approved counterpart from the same company may be discontinued.

One significant advantage of FDA approval of assays is the standardization of interpretive criteria. In the example of ALK rearrangement testing, the percentage of positive cells required to call a specimen positive would be standardized for the FDA assay, whereas a laboratory performing an in-house, non-FDA-approved assay may determine its own cutoff. Although the validity of individual cutoffs can be debated, standardization across laboratories certainly makes interlaboratory comparison more meaningful and simplifies the clinical interpretation of reports.

Break-Apart Chromogenic In Situ Hybridization

Recently, brightfield, dual-color, break-apart chromogenic in situ hybridization (CISH) assays have been described. Possible benefits of this technique include simultaneous assessment of tissue morphology while observing the relevant genetic abnormality and minimal signal quenching over time. Major limitations of this approach include reduced ability to discern the degree of split between 5' and 3' signals because of larger diameter signals and reduced visual contrast of signals compared with FISH. In a recent report, assuming that break-apart FISH represents the current gold standard, 5% (1 of 19) of ALK FISH-positive cases (determined using Vysis break-apart probes) were reported as negative using CISH.³⁶

Polymerase Chain Reaction

Several reverse transcriptase-polymerase chain reaction (RT-PCR)-based techniques for detecting *ALK* rearrangements exist.³⁷⁻⁴⁰ PCR of genomic DNA for fusion genes can be problematic, and most PCR techniques for detecting ALK rely on determining the presence of specific messenger RNA (mRNA) transcript.⁴¹ The presence and size of amplicons are evaluated, either with or without DNA sequencing, to rule out the possibility of false (ie, nonspecific) priming. Quantitative PCR assays are similar, except that detection of amplicons is measured after each PCR cycle using fluorescently labeled probes, and the cycle at which this reaction reaches a threshold level is recorded. Whereas earlier approaches required fresh-frozen tissue because of the known impact of formalin fixation in the degradation of RNA, newer commercially developed PCR techniques may overcome such limitations and allow accurate assessments from FFPE tissue.^{38,39,42}

Fusion partner-specific polymerase chain reaction primer sets—Fusion partner-specific PCR techniques have the ability to identify the exact fusion partner in an activating ALK rearrangement from FFPE tissue, and it has the potential to distinguish different breakpoints in the same 5' partner.^{38,39,42} Because of the variable length of *EML4* sequence combined with typically shorter RNA fragments derived from FFPE tissue, multiple *EML4* primers are used to minimize the amplicon size. Some fusion partners or breakpoint variants may be missed if the primers contained in the assay are not specifically designed to evaluate these alterations. When ALK FISH-positive cases were retrospectively analyzed for *EML4-ALK* transcripts by RT-PCR in the initial phase 1 study of crizotinib, transcripts could not be detected in 31% of cases.¹ Although some of these RT-PCR-negative cases may represent a failure of technique, they also may represent uncommon EML-ALK variants or non-*EML4* fusion variants that were being missed by the primer sets used in the assay. Similarly, in a Japanese study that examined the use of RT-PCR for *EML4-ALK*, 4 of 12 (33%) ALK FISH-positive samples were negative, raising further concerns regarding the sensitivity of PCR as a broad screening assay for ALK.⁴³ Finally, although the specificity of RT-PCR as a screening tool is likely to be extremely high, especially if the amplified regions of combinatorial DNA (cDNA) are sequenced, the dangers of cross-contamination, for example, from reuse of the same blade at sectioning, means that false-positive results may occur with this highly sensitive technique.

Kinase domain-only polymerase chain reaction primer sets—Quantitative RT-PCR ALK kinase domain assays exploit the observation that ALK is expressed at negligible levels in most normal adult lung tissues, and the existence of elevated levels of ALK expression may be indicative of pathologic ALK activation. In an assay now being marketed for commercial use, quantitative PCR of a region of ALK, corresponding to the intracellular kinase domain, compared with that of an endogenous internal reference gene is used (cytochrome c oxidase subunit 5B [*COX5B*]).⁴⁰ It remains unknown whether the expression

of native ALK in the brain will affect the validity of the test when applied to tissue from brain metastases.¹⁶

Immunohistochemistry

Immunohistochemical (IHC) analysis for ALK, which also capitalizes on the minimal expression of native ALK in most normal tissues,⁶ is another potential method of detection, and various antibodies are currently in commercial development (Table 2).^{44,46,47} Although IHC is now the dominant ALK screening methodology in lymphomas because of the high level of expression observed with the common 5' partners in ALK-positive non-Hodgkin lymphoma, in other ALK-positive diseases, including NSCLC, the expression level of the fusion protein is lower, and the development of routine IHC has been more problematic.^{44,45,50} In addition, it should be noted when reviewing the available literature on IHC for ALK in NSCLC that a single, uniform technique, or comparator, has not been evaluated. Instead, there is significant variance in the antibody being considered; the number and type of specimen being examined; which antigen retrieval, antibody detection, and amplification techniques were used; which scoring technique was used; what "gold standard" was used for comparison; and how intensively the reliability and reproducibility of the overall technique across different laboratories and observers was examined. If only FISH-positive cases are assessed in a study, then, although the sensitivity of a second technique like IHC can be determined, its specificity cannot.^{1,45} When mixtures of FISH-positive and FISH-negative cases are assessed, both sensitivity and specificity may be determined, but the degree of enrichment of the cohort being tested for factors associated with ALK positivity may significantly affect the transferability of the results to differently selected/unselected populations. For example, if only reasonable sized studies that compare IHC versus break-apart FISH testing are considered, then the cohorts explored range from unselected, consecutive case series comprising approximately 70% adenocarcinoma to series that only assessed either adenocarcinomas occurring in never-smokers or nonsquamous cancers known to be epidermal growth factor receptor (EGFR) wild-type/EGFR tyrosine kinase inhibitor nonresponders (Table 2).^{47,48} When different antibodies have been compared head-to-head in NSCLC, D5F3 (Cell Signaling Technologies, Beverly, Mass) appeared to be both more sensitive (100% vs 67%) and more specific (99% vs 97%) than the ALK1 antibody (DAKO, Carpinteria, Calif).⁴⁴ In contrast, studies using other antibodies have reported increased sensitivity through the use of several different signal amplification steps.^{24,45,49} Semiautomated quantification using an Aperio-derived score intensity (the average absorbance of pathologist-determined positive areas multiplied by the percentage of area staining above baseline) has been used,⁴⁴ but most of the scoring systems reported have used a visual, semiquantile range (0 to 3+), with the fine details of how each scoring category within the range is defined varying between studies (Table 2).⁴⁴ At its best, IHC has reported sensitivity of 100% and specificity of 97% compared with FISH testing, and the IHC kappa value for interobserver agreement is 0.94.⁴⁴ However, sensitivities as low as 67% and interobserver agreement as low as 0.5 also have been reported.^{44,46}

Consequently, it would appear that, although IHC already seems to perform sufficiently well in some expert centers to allow it to be considered as a primary screening tool, standardization of IHC to reliably reproduce the results of such high-performing centers across multiple sites is required. Numerous technical steps in tissue preparation and processing and IHC procedures are subject to laboratory-to-laboratory variability, including preprocessing fixation time, fixation parameters, specific chemical composition of processing and embedding reagents, type of antigen retrieval performed, heating device for antigen retrieval, primary antibody incubation time, detection system, and counterstaining method.⁵¹ Consistent with the potential for sample processing before IHC to affect outcomes, even when multiple samples from the same patient have been assessed in the

same center, variation in IHC signal scoring from positive, through equivocal, to negative has been reported, despite the persistence of FISH positivity.⁴⁴ Should an antibody gain FDA approval for ALK testing, acceptable ranges for many of these different parameters probably would be specified. The most notable previous example relating to the challenges of IHC standardization is in the evaluation of breast human epidermal growth factor receptor 2 (HER2) in breast cancer.^{52,53} Even after significant experience with HER2 IHC testing, multiple studies have highlighted continuing interlaboratory discordance rates of up to 35%.⁵⁴⁻⁵⁶ However, if test variability and interpretation can be addressed, then the potentially lower cost and greater ease of performing the test eventually may lead to widespread use of the assay beyond key expert centers, either alone or as a screening test before FISH testing (see above).

Next-Generation Sequencing

Next-generation sequencing (NGS) refers to a variety of different platforms that allow for parallel sequencing of multiple analytes simultaneously. The use of NGS for detecting gene copy number alterations and rearrangements is feasible, and this broad discovery approach already has been used to identify several novel ALK rearrangements and other relevant fusion genes in NSCLC.^{9,57-60} However, adoption, particularly of quicker, focused approaches, examining only specific panels of genes, is growing in the clinical setting. Like in PCR-based approaches, if a focused approach is taken, then fairly detailed knowledge of what is being searched for is required to avoid missing a significant number of rearrangements. Perhaps the major potential advantage of using an NGS approach is that a well designed panel may be able to provide information on a significant number of the known gene mutations, gene rearrangements, and copy number alterations across multiple different cancers within a single multiplexed assay, minimizing the decision-making required for clinicians or pathologists when considering which test to order for each patient. Thus, as the amount of tissue required and the cost of NGS decreases and clinical laboratories and regulatory bodies increasingly validate these platforms, the convenience and cost-effectiveness of screening for multiple different abnormalities in populations at the same time (as opposed to running multiple, discrete assays) is likely to drive the wider adoption of this approach.⁶¹ There are multiple different NGS platforms, such as PacBio RS (Pacific Biosciences, Menlo Park, Calif), Ion Torrent PGM (Life Technologies, Carlsbad, Calif), and Illumina HiSeq (Illumina, San Diego, Calif)⁶²; however to our knowledge, there are no large data series that allow comparison of ALK detection between these platforms and the current gold standard of break-apart FISH testing.

Other Techniques

Because ALK fusions represent abnormal proteins that do not occur in nontransformed tissues, proteomic-based approaches potentially applicable to small volumes of many different body fluids or tissues examining for the presence of specific fusions have been reported but are not commercially available to date.^{19,63}

Rationalizing the Differences Between ALK Detection Techniques

Given the high clinical benefit reported in ALK FISH-positive patients who received treatment with crizotinib, we must assume that break-apart FISH testing represents the current gold standard for detecting ALK positivity. When considering any other technique, several questions arise. First, how reliably do these other techniques capture the same FISH-positive population? Specifically, what is their sensitivity and specificity for detecting these same cases across different laboratories and different observers? In the most simplistic assessment, any technique with reproducible 100% sensitivity and specificity may be a reasonable alternative to break-apart FISH testing, with preference for 1 test over the other being based on the feasibility/expertise of any chosen provider and the costs to the payer.

However, what happens if a new test detects less than the number of FISH-positive cases? Are these false-negative results, or could the new assay be even more accurately delineating those patients who will derive clinical benefit from crizotinib than the FISH test, given that not 100% of FISH-positive patients will benefit from crizotinib?^{1,28} Similarly, if an alternative methodology detects more than the number of FISH-positive cases, then are these false-positive results or a new population set to benefit from crizotinib that previously was missed (Fig. 2)? Currently, we do not know the answers to these questions. Detailed clinical outcomes from crizotinib therapy are only available from patients enrolled on clinical trials, all of whom were proven to be ALK-positive using the Vysis break-apart FISH assay.^{1,28} However, within well constructed studies in the future, examining the benefit from crizotinib in patients who are prospectively known to be positive or negative by multiple different ALK detection assays, we should be able to start to address these issues directly.

It is possible that, ultimately, a single test may not be adequate. Sometimes, 1 technique may be preferable over another on a case-by-case basis, depending on the specimen available. For example, decalcified bone specimens may be more appropriate for IHC than for FISH.^{24,26,49} Two-tiered approaches using 1 primary assay and a different secondary assay to confirm or deny the true ALK nature of equivocal cases already have been proposed. IHC or RT-PCR could be used to explore FISH equivocal cases on the basis of either cell counts close to, but below, the cutoff point for determining tumor positivity or with atypical negative cytogenetic patterns.^{31,45} More commonly, however, as with Her2, 2-tiered testing has been proposed using IHC as a potentially more convenient and/or cheaper primary assay, reserving FISH as potentially a more stringent but more labor-intensive and/or more expensive second assay for equivocal IHC cases.^{46,47,53,54} This approach rests on emerging data that, for several different antibodies and scoring techniques, completely negative IHC cases appear to be true-negatives with respect to FISH and that strong IHC-positive cases (eg 3+) tend to be uniformly FISH-positive.⁴⁶⁻⁴⁹ Therefore, confirmatory ALK FISH testing would only need to be applied as a second screen to capture the small numbers of missed “true” ALK-positive cases by testing, for example, either only IHC 2+ cases or both 2+ and 1+ cases (Fig. 3). When such a scenario is modeled, because of differences in the techniques used and in the degree of preselection of the cohorts assessed, the proportions of true ALK-positive specimens that fall within any “equivocal” IHC group vary significantly between studies (Table 2). For example, estimates of FISH positivity rates among IHC 2+ cases range from 33% to 85%.⁴⁶⁻⁴⁸ To determine the potential cost saving in practice of a 2-tiered approach, the absolute size of the 1+ and 2+ groups would have to be known.^{46,48} In addition, because only the break-apart FISH assay is currently FDA approved, the true cost of a reliable IHC assay to accurately inform cost-effectiveness calculations currently remains unknown. However, to illustrate the potential of a 2-tiered approach, combining 2 recent ALK IHC articles together to include 554 screened cases in total, a combined strategy may entail FISH screening 48 IHC 2+ cases and 1+ cases to identify 5 additional ALK FISH-positive cases.^{46,47} At its most simplistic, in this example, such a tiered approach may be cheaper but as effective as using FISH on everyone, provided that a validated IHC assay was at least 9% (48 of 554) less expensive than the FISH assay (cost of IHC assay = X, cost of FISH assay = Y, with overall cost saving to the 2-tiered approach if $554 Y > 554 X + 48 Y$). Alternatively, if payers are not committed to finding every “true” ALK-positive case but would consider adopting approaches with <100% sensitivity if they are significantly cheaper, then the cost of the life-years lost and gained from such approaches would have to be calculated and the payers’ acceptability of these costs determined.^{50,61}

In conclusion, the approval of crizotinib offers a new specific therapeutic option for patients with ALK-positive NSCLC. Maximizing the appropriate use of crizotinib depends on the deployment of assays for detecting ALK positivity that are accurate, reproducible across multiple centers, and scalable. Currently, break-apart FISH is the only testing methodology

recommended for ALK testing within the draft College of American Pathologists/ International Association for the Study of Lung Cancer/Association of Molecular Pathology guidelines.⁶³ PCR, IHC, and (potentially) NGS tests are now also available locally and, in some cases, commercially and offer some advantages and disadvantages. Factors like changing costs and local experience may influence the initial choice of assay. However, future studies will be required to directly compare and contrast these different testing methodologies in relation to predicting benefit from crizotinib.

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Figure 1.

This is a schematic of major anaplastic lymphoma kinase (*ALK*) testing methodologies. (a) Note that reverse transcriptase-polymerase chain reaction (RT-PCR) focused solely on the *ALK* kinase domain, searching for a threshold signal in a quantitative assay, is not fusion partner-specific; whereas RT-PCR that spans the common breakpoint in rearrangements is fusion partner-specific and searches for the presence/absence of a generated amplicon. *EML4* indicates echinoderm microtubule-associated protein like 4; *EML4 v1* and *EML4 v2* refer to two of the possible variants of *EML4*. Break-apart fluorescence in situ hybridization (FISH) testing is not fusion partner-specific. IHC is also not strictly fusion partner-dependent. However, because transcriptional activity is set by the promoter/enhancer of the 5' fusion partner, and different fusion proteins may have both different intracellular locations (because of an association between the 5' oligomerization domains in the fusion protein with the native form of the 5' partner as well as with the rearranged form) and, along with different fusion variants, potentially different half-lives, the absolute protein expression levels and patterns of staining with IHC may vary, depending on the exact *ALK* fusion and/or variant present.¹⁹⁻²¹ Techniques not presented here include FISH testing using fusion probe sets, chromogenic in situ hybridization, and next-generation sequencing. (b) Different

break-apart FISH testing patterns include (clockwise from top left) fused (negative), single green (negative), split (positive), and single red (positive).

Figure 2.

Hypothetical comparisons are illustrated for different diagnostic techniques versus a biologically “true” anaplastic lymphoma kinase (*ALK*)-positive patient population. Definitive determination of the validity of techniques with higher or lower positivity rates will depend on head-to-head clinical comparisons entailing the expansion of access to *ALK* inhibitors beyond only patients who have positive results from break-apart fluorescence in situ hybridization (FISH) analysis.

Figure 3.

This schematic illustrates a possible 2-tier testing strategy for detecting anaplastic lymphoma kinase (*ALK*) using immunohistochemistry (IHC) and confirmatory ALK fluorescence in situ hybridization (FISH) for IHC equivocal cases. For the purposes of this illustration, an IHC staining score of 3+ would be considered true-positive, and an IHC score of 0 would be considered true-negative. The resource and cost-effectiveness implications of this approach will depend on the ultimate cost of a validated IHC assay, the absolute number of cases falling within the equivocal category, and the proportion of cases that are FISH-positive residing within the equivocal category. These equivocal category variables will be affected both by the specific IHC assay used and by any aspects of patient enrichment applied to the tested population.

TABLE 1

Pros and Cons of the Major Different Anaplastic Lymphoma Kinase Testing Methodologies in Non-small Cell Lung Cancer

Testing Method	Pros	Cons
Break-apart FISH	Detects <i>ALK</i> rearrangements regardless of variant and fusion partner; allows use of archival FFPE tissue.; requires only ~100 tumor cells; is clinically validated, associated with outcome studies; is FDA-approved for clinical application	Requires fluorescent microscope; interpretation requires specialized training; high cost of validated test; does not identify the specific fusion partner or breakpoint variant; may miss rare, complex rearrangements
RT-PCR (fusion partner-specific)	Highly specific; identifies the specific 5' fusion partner and breakpoint variant (the significance of which remains under investigation)	May miss atypical <i>ALK</i> variants, or fusion partners, depending on assay design; testing in FFPE requires skillful application of the technique because of degradation of nucleic acids; cost of validated test for use in routine pathology laboratories is unknown, although commercial providers exist
qPCR (for kinase domain)	qPCR of <i>ALK</i> kinase domain allows identification of <i>ALK</i> even if unknown fusion partner	Testing in FFPE requires skillful application of the technique because of degradation of nucleic acids; cost of validated test for use in routine pathology laboratories is unknown, although commercial providers exist
IHC	In theory, detects <i>ALK</i> rearrangements regardless of variant and fusion partner; however, fusion partner and variant may influence protein levels and location; preferentially detects higher levels of expression that may (or may not) be associated more with outcome from specific intervention; allows use of archival FFPE tissue; requires only a small amount of tissue, but the exact number of cells needed for reliability of assay has not been determined; technical infrastructure is already available in many laboratories for adoption; potentially rapid turn-around time and cost of standard (non-FDA approved) consumables and equipment for routine use is low	Requires standardization of reagents and protocols across pathology laboratories; cost of validated test unknown; no internal positive controls for staining are available

Abbreviations: *ALK*, anaplastic lymphoma kinase gene; FDA, US Food and Drug Administration; FFPE, formalin-fixed, paraffin-embedded; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction.

TABLE 2

Studies Exploring Immunohistochemical Detection of Anaplastic Lymphoma Kinase Positivity in Non-small Cell Lung Cancer Compared With Break-Apart Fluorescence In Situ Hybridization Testing

Study	Antibody (Supplier)	“Gold-Standard” Comparator ^a	No. of Tumors Screened ^b	IHC Scoring System	No. of ALK-Positive Patients Identified by Comparator	No. of ALK-Positive Patients Identified by IHC Category	Kappa Statistic			Specificity, %
							Interobserver Agreement	Intraobserver Agreement	Sensitivity, %	
Mino-Kenudson 2010 ⁴⁴	D5F3 (Cell Signaling, Beverly, Mass)	FISH	153	By eye: 0 to 3+ ^c	22	NR	0.94	NR	100	99
Mino-Kenudson 2010 ⁴⁴	ALK1 (Dako, Carpinteria, Calif)	FISH	153	By eye: 0 to 3+ ^c	22	NR	0.79	NR	67	97
Rodig 2009 ⁴⁵	ALK1 with/without tyramide amplification (Dako)	FISH	358	By eye: Positive or negative	20 (10 available for IHC assessment)	4 of 10 FISH-positive cases identified as IHC-positive without tyramide amplification; 8 of 10 identified with tyramide amplification	NR	NR	80	NA (only FISH-positive cases were tested)
Yi 2011 ⁴⁶	ALK1 (Dako)	FISH	101	By eye: 0 to 3+ ^d	10	8 of 8 IHC 3+, 1 of 3 IHC 2+, 1 of 21 IHC 1+, and 0 of 69 IHC-negative cases were FISH-positive	0.50-0.80	0.82	90	98
Paik 2011 ⁴⁷	Clone 5A4 (Novocast Laboratories, Ltd., Newcastle Upon Tyne, United Kingdom)	FISH	640	By eye: 0 to 3+ ^e	28	22 of 22 IHC 3+, 6 of 16 IHC 2+, 0 of 16 IHC 1+, and 0 of 586 IHC-negative cases were FISH-positive	0.94	NR	100	96
Park 2012 ⁴⁸	Clone 5A4 (Novocast)	FISH	262	By eye, 0-3+ ^f	25	9 of 9 IHC 3+, 11 of 13 IHC	NR	NR	100	99

Study	Antibody (Supplier)	"Gold-Standard" Comparator ^a	No. of Tumors Screened ^b	IHC Scoring System	No. of ALK-Positive Patients Identified by Comparator	No. of ALK-Positive Patients Identified by IHC	Kappa Statistic		
							Interobserver Agreement	Intraobserver Agreement	Sensitivity, %
						2+, 5 of 6 IHC 1+, and 0 of 234 IHC-negative cases were FISH-positive			Specificity, %
Sakairi 2010 ²⁴	Clone 5A4 (Abeam, Boston, Mass)	FISH	109	By eye: Positive, suspicious, or negative ^d	7	6 of 6 "positive" cases and 1 of 17 "suspicious" cases were FISH-positive; IHC-negative cases were not tested by FISH	NR	NR	NR
McLeer-Florin 2012 ⁴⁹	Clone 5A4 (Abeam) with amplification kit (Ventana, Tucson, Ariz)	FISH	441, Including 100 (IHC-positive or EGFR and KRAS WT) that were tested by FISH	By eye: Percent-age of positive cells, 0 to 3+	19 of 100	19 of 20 "Positive" cases, 2 of 2 "doubtful" cases, and 0 of 59 IHC-negative cases were FISH-positive	NR	NR	100
									95

Abbreviations: ALK+, positive for an anaplastic lymphoma kinase (ALK) gene rearrangement; EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; NA, not applicable; NR, not reported; NSCLC, non-small cell lung cancer; WT, wild type.

^a Break-apart FISH assay used minor nonstandard (per US Food and Drug Administration assay) criteria⁴³; break-apart and fusion FISH assays used >50% as positive.²⁴

^b The tumors screened were adenocarcinomas.^{44,49} Screened adenocarcinomas included 131 patients enriched for factors associated with EGFR mutations, such as young age and low/no smoking status⁴⁵; never-smokers only⁴⁶; a consecutive series of patients with NSCLC who underwent resection (70% adenocarcinomas)⁴⁷; patients with nonsquamous NSCLC and known EGFR WT or EGFR tyrosine kinase inhibitor nonresponders (69% adenocarcinomas)⁴⁸; and patients who had hilar/mediastinal lymph nodes sampled by endobronchial ultrasound (75% adenocarcinomas).²⁴

^c In this study, positive indicated any staining intensity over background in 10% of tumor cells.⁴⁴

^d In this study, 3+ indicated intense, granular cytoplasmic staining; 2+, moderate, smooth cytoplasmic staining; 1+, faint cytoplasmic staining in 10% of tumor cells.⁴⁶

^e In this study, 3+ indicated strong staining intensity in >5% of cells; 2+, moderate staining intensity in >5% of cells, faint or weak staining intensity in >5% of cells, or any staining intensity in 5% of cells.⁴⁷

^fIn this study, 3+ indicated strong, granular cytoplasmic staining; 2+, moderate cytoplasmic staining; 1+, faint cytoplasmic staining in 10% of tumor cells in resected specimen or 90% of cells in biopsy specimen.⁴⁸

^gIn this study, positive indicated strongly positive for fine, granular cytoplasmic staining; negative, no staining; suspicious, weak staining.²⁴

^hIn this study, 3+ indicated intense, granular cytoplasmic staining; 2+, moderate cytoplasmic staining; 1+, faint cytoplasmic staining; doubtful cases demonstrated staining of any intensity in <30% of cells.⁴⁹