Editorial

Low level ALK FISH positive results should be approached with caution and confirmed by an orthogonal method

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The anaplastic lymphoma kinase gene (*ALK*) is located on 2p23 and encodes a transmembrane receptor tyrosine kinase (RTK) that belongs to the superfamily of insulin receptors. In normal physiology, ALK is activated by endogenous ligands Pleitrophin (PTN) and Midkine (MK), which play a role in fetal brain development. The binding of these ligands leads to dimerization and intracellular tyrosine kinase (TK) activation, initiating a signal cascade through several canonical signaling pathways (1).

In 1994, Morris *et al.* (2) first described the *ALK* rearrangement in anaplastic large cell lymphoma, which led to the gene being named *ALK* (i.e., anaplastic lymphoma kinase). In 2007, Soda *et al.* (3) identified the first *ALK* rearrangement in non-small cell lung carcinoma (NSCLC) The echinoderm microtubule-associated protein-like 4 (*EML4*) is the fusion partner in 75% of cases, but about 90 different fusion partners have been described for *ALK* gene in NSCLC (4).

Genomic rearrangements that involve the *ALK* gene result in the formation of a chimeric fusion protein. This fusion protein containing ALK tyrosine kinase domain is under the control of the promoter of ALK fusion partner and this leads to ligand-independent dimerization and constitutive tyrosine kinase activity (5). Neoplasms with *ALK* fusions are highly dependent on constitutive ALK tyrosine kinase activity, which makes them highly sensitive to ALK tyrosine kinase inhibitors (TKIs). As of April 2023, there are four generations of eight ALK TKIs either with United Stated Food and Drug Administration (FDA) approval or in clinical testing (5). Identifying neoplasms with ALK rearrangements is crucial for the administration of ALK TKIs.

Detection of *ALK* rearrangements by fluorescence in-situ hybridization (FISH) is a poster child for companion diagnostics. The first-generation ALK inhibitor Crizotinib (Pfizer) and Vysis ALK break-apart FISH (Abbot Technologies) obtained FDA approval simultaneously in 2011 (6). For a long time, FISH was the sole gold standard for ALK biomarker testing. However, the 2018 Guideline for the Selection of Lung Cancer Patients for Treatment with Targeted Tyrosine Kinase Inhibitors from College of American Pathologists (CAP)-International Association for the Study of Lung Cancer (IASL) and Association of the Molecular Pathology (AMP) (7) established ALK based immunohistochemistry equivalent and an alternative to *ALK* FISH testing. Nonetheless, FISH testing for *ALK* is still considered one of the first-line testing strategies. As of April 2023, the Vysis ALK Break-apart FISH is still indicated as an aid in identifying patient eligible for treatment with the drug label of Crizotinib and Brigatinib in accordance with therapeutic labeling in the United States (6,8).

Compared to other FISH tests performed in clinical laboratories, *ALK* FISH testing is unusual in that it involves

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a two-step process. In the first step, at least 50 cells are evaluated. If fewer than five cells (10%) have a positive FISH pattern (i.e., split pattern and/or deletion of 5'*ALK*), the results are interpreted as negative. Conversely, if more than 25 cells (50%) have a positive pattern, the results are considered positive. If 5–25 cells (10–50%) have a positive pattern, the result is considered equivocal, and an additional 50 cells are analyzed. If 5–25 cells (10–50%) have a positive pattern in the second analysis, the result is considered equivocal. The percentage of positive cells is calculated by adding the positive cells from the first and second cell counts and dividing by 100. A result is interpreted as positive if there are ≥15% positive cells; otherwise, the result is considered negative (6).

Due to its FDA-approved companion diagnostic status, the *ALK* FISH cut-off value is rigid. It should be noted that *ROS1* and *RET* break-apart FISH are different from *ALK* break-apart FISH, as these probes do not have FDA-approved companion diagnostic assay status, providing clinical laboratories with more leeway in terms of validation, establishing cut-off values, and slide interpretation. Usually, clinical laboratories in the United States establish their cut-off values following American College of Medical Genetics (ACMG) guidelines (9).

Currently, according to the 2018 CAP-IASL-AMP Lung Cancer Testing Guidelines, FISH and IHC are considered the gold standard for ALK testing (7). Although not directly addressed in the guidelines, DNA and RNA-based next-generation sequencing (NGS) methods are increasingly being utilized, and discrepancies between these different methodologies and FISH are observed for some cases. Typically, discrepancies, such as false-positive FISH results, are observed with low levels (15–25%) of *ALK* FISH positivity (10-13). Interestingly, a couple of studies have shown an association between low FISH positivity and a low response rate to ALK inhibitors (14-16).

In their meticulous study, van Gulik *et al.* (17) demonstrated the confounding role of polyploidy in testing for ALK rearrangement (and to some extent *ROS1*) by break-apart FISH. The study showed that larger polyploid nuclei can lead to false positive results, particularly when the number of positive cells is low. The study also highlighted the potential role of section thickness as a confounding factor for false positivity. Although false positive *ALK* FISH results are a well-known phenomenon for low positive tumors, this study is novel in its association between polyploidy and false positivity. This issue can be applied to other break-apart FISH probes, but it is particularly significant for the *ALK* break-apart probe as clinical laboratories do not have the flexibility to establish their own cut-off values if they want to implement the test according to FDA specifications.

Although the impact of polyploidy on false positive FISH results needs to be confirmed by additional studies from different groups, the potential confounding role of polyploidy is highly intriguing. Polyploidy rates differ significantly between tumor types, with germ cell tumors having a rate of 58%, while only 5% or fewer non-Hodgkin lymphomas exhibit polyploidy. NSCLC is among the neoplasms with the highest frequency of polyploidy, with a rate of 35% (18).

For NSCLC, multiple biomarker assays compete for limited tissue material. With the increasing prevalence of DNA and RNA-based next-generation sequencing (NGS) assays, FISH testing for NSCLC may become obsolete. Liquid biopsy testing of circulating nucleic acids is also increasingly used as a surrogate for tissue-based molecular testing. However, *ALK* and *ROS1* break-apart FISH are still considered among the first line of testing. Therefore, the technical limitations of FISH with break-apart probes are still a pertinent research and clinical practice point.

The study by van Gulik *et al.* (17) emphasizes the need for clinical laboratories to perform their own validation and establish their own analytical cutoffs based on the tissue/disease they intend to test. FISH testing is both an art and a science, and cookie-cutter solutions for result interpretation, such as universal FISH cutoffs, should be approached with caution. Borderline positive results should be confirmed by alternative technologies like IHC or NGS as low positive FISH results by break-apart probes may be false positives. Therefore, it is important for laboratories to be aware of the specifics of the tissue/disease they are testing and to perform validation and cutoff establishment accordingly.

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