Pharmacogenomics and Personalized Medicine

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REVIEW

# ALK (D5F3) CDx: an immunohistochemistry assay to identify ALK-positive NSCLC patients



This article was published in the following Dove Press journal:
Pharmacogenomics and Personalized Medicine

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**Abstract:** Screening for anaplastic lymphoma kinase (*ALK*) rearrangements is a very important process in treatment decision making for advanced non-small-cell lung cancer (NSCLC). Although fluorescent in situ hybridization (FISH) is considered the universally accepted reference standard, it is associated with technical difficulties and high costs that have made global implementation of this assay challenging. Conversely, ALK immunohistochemistry has shown high sensitivity and specificity compared to FISH and other molecular assays and is more cost-effective. In fact, the ALK (D5F3) CDx immunohistochemistry assay was approved by the US Food and Drug Administration as a standalone test for *ALK* rearrangements in lung cancer in 2015. In this review, we will discuss the overview of *ALK* rearrangements in NSCLC, various testing methods for *ALK* rearrangements, and the details of immunohistochemistry for ALK, in particular one with the ALK antibody clone D5F3.

**Keywords:** anaplastic lymphoma kinase gene *ALK*, D5F3 antibody, ALK (D5F3) CDx, non-small-cell lung cancer, adenocarcinoma

## *ALK* rearrangements in NSCLC

Molecular targeted therapy has brought a paradigm shift in treatment for advanced non-small-cell lung cancer (NSCLC). It has been shown that NSCLC patients with driver mutations have better progression-free survival (PFS) and overall survival with first- and second-line therapies compared to those with no treatable driver mutations.[1] After Lynch et al identified epidermal growth factor receptor (*EGFR*) mutations in tissue samples from NSCLC patients who had responded to gefitinib,[2] EGFR tyrosine kinase inhibitors (TKIs) have become innovative therapeutic agents in the field of lung cancer. As researchers strove to find new driver mutations, fusions of the echinoderm microtubule-associated protein-like four gene (*EML4*) and the anaplastic lymphoma kinase gene (*ALK*) in NSCLC patients were first reported in 2007 by Soda et al.[3]

*EML4–ALK* fusions are derived from inversions within the short arm of chromosome 2, and several *EML4–ALK* variants classified by the site of fusion have been reported.[4] *ALK*-rearranged tumors comprise 3%–7% of NSCLCs,[1,4,5] and the vast majority harbor *EML4–ALK* fusions, while rare fusion partners, such as *KIF5B*, *TFG*, *KLC1*, *HIPI*, *ASXL2*, *ATP6V1B1*, *PRKAR1A,* and *SPDYA*, have also been reported.[6–8] Clinically, *ALK* rearrangement-positive NSCLCs are typically seen in never or light smokers, of younger age, and harboring wild-type *EGFR* and *KRAS*.[9–12] Pathologically, most *ALK* rearrangement-positive NSCLCs exhibit adenocarcinoma histology; solid pattern with signet cells and/or mucinous cribriform pattern are often seen, at least focally, in these tumors.[6,10,12,13]

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Dovepress
http://dx.doi.org/10.2147/PGPM.S156672

## Treatment for *ALK*-rearranged NSCLC

*ALK* rearrangement-positive NSCLCs are highly sensitive to ALK-TKIs. Shaw et al conducted a Phase III study and showed that crizotinib, a first-generation ALK TKI, had better response rate and longer PFS compared to pemetrexed or docetaxel in previously treated *ALK* rearrangement-positive NSCLC patients (65% vs 20% and 7.7 vs 3.0 months, respectively).[14] The PROFILE 1014 Phase III study compared crizotinib with pemetrexed plus carboplatin/cisplatin in treatment-naïve *ALK* rearrangement-positive lung cancer patients, and again showed better response rate and longer PFS (74% and 45% and 10.9 vs 7.0 months, respectively).[15] Interestingly, patients with ALK variant 1 were more responsive to crizotinib than those with non-variant 1.[16] Alectinib, a second-generation ALK TKI, showed better PFS compared to crizotinib in untreated *ALK* rearrangement-positive NSCLC in two Phase III studies, the one in a Japanese population (the J-ALEX trial)[17] and the other in a worldwide population (the ALEX trial).[18] Ceritinib, another second-generation ALK TKI, showed longer PFS in treatment-naïve *ALK* rearrangement-positive NSCLC patients compared to platinum-based chemotherapy,[19] and in patients after development of resistance to crizotinib compared to chemotherapy (the ASCEND-5 trial).[20] A Phase II study of lorlatinib, a third-generation ALK TKI, resulted in an objective response rate of 59% in *ALK* or *ROS-1* rearrangement-positive NSCLC patients, most of whom had previously been treated with ALK TKIs.[21] Lorlatinib was granted breakthrough therapy status in the United States based on these results.

## Detection of *ALK* rearrangements in lung cancer

Fluorescent in situ hybridization (FISH) is considered as the universally accepted reference standard for detection of *ALK* rearrangements, and the Vysis LSI ALK Break Apart FISH Probe Kit (Abbott Molecular Inc., Des Plaines, IL, USA) was approved by the US Food and Drug Administration (FDA) as the first screening method for *ALK* rearrangements in lung cancer. The Vysis LSI ALK Break Apart FISH Probe Kit consists of two probes, Vysis LSI 3′-ALK (Orange) and Vysis LSI 5′-ALK (Green). In the normal condition (without rearrangements), two signals (red/green) appear to be overlapped or fused leading to a yellow signal due to their proximity. However, under the 2p23 *ALK* rearrangement, the red and green signals are apart with some distance (two or more times the diameter of the largest signal) or red-only signals may be seen when the nonfunctioning 5′ side of *ALK* gene is eliminated upon rearrangement.[22] To minimize technical bias, a two-step assessment strategy with two independent reviewers is recommended. The first reviewer scores 50 tumor cells. If the split pattern and/or isolated 3′ (red) pattern are seen in <10% of the examined tumor cells, the tissue sample is considered negative for an *ALK* rearrangement; a rate greater than 50% is considered positive; and a rate of 10%–50% is considered equivocal. In the latter situation, a second independent reviewer evaluates an additional 50 tumor cells, and a final rate of tumor cells with the positive signal patterns is calculated based on the sum of the first and second scores. The specimen is then classified based on the final rate with the cutoff of 15%.[8] However, there are several preanalytical and analytical issues that may result in false negative or false positive interpretation of FISH.[8,23,24] First, inadequate fixation and storage could cause false negative results.[8] Second, *ALK* (2p23.2) is located close to *EML4* (2p21) on the same chromosome arm; thus, the spilt signals in NSCLC with an *EML4–ALK* fusion could be erroneously interpreted as fused signals leading to false negative results. Third, normal cells could be interpreted as tumor cells in the dark field and dilute the rate of positive cells resulting in false negative results. Fourth, the rate of tumor cells with break-apart or isolated red signals falls within the range of 10%–20% in approximately 5%–10% of NSCLCs.[25–28] Such equivocal counts represent one of the major sources of false positive or false negative results[26–28] and discordant results between the observers.[29] Therefore, an external quality assessment is extremely important for *ALK* FISH testing.[30] In addition, small biopsy specimens, including transbronchial lung biopsy, endobronchial ultrasound-guided transbronchial needle aspiration, or computed tomography-guided transthoracic needle biopsy, may not provide enough tumor cells for FISH analysis because at least 50 more tumor cells need to be evaluated.[31]

Real-time polymerase chain reaction (RT-PCR) is another method used for diagnosis of *ALK* rearrangement-positive NSCLC. Takeuchi et al[32] showed that RT-PCR had 100% sensitivity and specificity for *EML4–ALK* rearrangement-positive NSCLC diagnosed by FISH. Several studies revealed high concordance between RT-PCR and FISH/immunohistochemistry (IHC), with 94%–100% sensitivity and specificity.[8] However, high-quality RNA is required for RT-PCR, and formalin-fixed paraffin-embedded (FFPE) specimens are usually inappropriate for RT-PCR. In addition, we need to know exact fusion partners to design primers for RT-PCR; thus, *ALK* rearrangements with unknown/novel partners will not be captured by this method.[8]

Next-generation sequencing (NGS) is an emerging technology that allows examination of millions or billions of DNA strands in parallel. NGS can examine a large panel of driver mutations simultaneously, and requires a smaller volume of specimen compared to sequential analyzing for driver mutations such as *EGFR*, *ALK*, *ROS1*, *RET*, and *BRAF*. There were two main types of NGS, DNA-based NGS and hybrid capture-based NGS. DNA-based NGS could assess for already known and designed breakpoints.[8] A recent study from Europe showed the sensitivity and specificity of DNA-based NGS using the Oncomine Solid Tumor Fusion Transcript Kit (Thermo Fisher Scientific, Waltham, MA, USA) for *ALK* rearrangement-positive NSCLC diagnosed by FISH and IHC as 85% and 79%, respectively.[33] On the contrary, hybrid capture-based NGS could analyze most breakpoints, even if they are unknown. Drilon et al[34] performed hybrid-captured NGS on lung adenocarcinomas from patients with a ≤15 pack-year smoking history and without 11 major driver mutations and fusions including *EGFR*, *ALK*, and *ROS1* by conventional (non-NGS) molecular testing. They were successful in detecting *SOCS5-ALK* and *HIP1-ALK*, and concluded that hybrid capture-based NGS was comprehensive and efficient. However, turnaround time for NGS is typically 2 weeks or longer and that may be too long for patients with advanced NSCLC to wait.[35,36] Needless to say, NGS is much more expensive than other methods at this time.

## ALK IHC in lung cancer

Because of technical difficulties and/or higher costs of FISH, RT-PCR, and NGS, IHC is increasingly used to detect *ALK* rearrangements. There are four ALK antibody clones that have been evaluated for NSCLC. They are ALK1, 5A4, D5F3, and anti-ALK(1A4). The clone ALK1 (Dako, Carpinteria, CA, USA) that recognizes the c-terminal of ALK tyrosine kinase does not have enough sensitivity to detect often weak ALK protein expression secondary to *ALK* rearrangements in NSCLC.[37] The sensitivity and specificity of IHC with the clone ALK1 (1:2) and EnVison+ detection system (Dako) in detecting *ALK* rearrangement-positive NSCLC diagnosed by FISH were 67% and 97%, respectively.[38] The 1A4 anti-ALK antibody (Origene Technologies Inc., Rockville, MD, USA), a recombinant protein that recognizes amino acids 426–528 of the 680 NPM-ALK protein, has been shown to have great sensitivity (100%), but low specificity (70%) (although no details in the IHC protocol were provided).[39] Thus, screening with the anti-ALK antibody may result in a high false positive rate.

Conversely, IHC with the clone 5A4 or D5F3 has good sensitivity and specificity for *ALK* rearrangements in NSCLC and can be used as a screening method.[40,41] Paik et al[42] and Kim et al[43] used the clone 5A4 (1:30; Novocastra, Newcastle, UK) and iVIEW detection system (Ventana Medical Systems Inc., Tucson, AZ, USA) for ALK IHC with four-tiered scoring system (0–3+), and reported 100% and 100% sensitivity, and 96% and 98% specificity, respectively, with >2+ as positive. Similarly, the clone 5A4 produced by Abcam (Cambridge, UK) has shown sensitivity and specificity of 100% for *ALK* rearrangement-positive NSCLC with FISH as the gold standard.[44] In this study, to increase the sensitivity of detection, the intercalated antibody enhanced polymer (iAEP) as well as EnVison FLEX+ detection system (Dako) were used for IHC with the antibody clone (1:100). However, the performance of this antibody (clone 5A4; Abcam) may not be optimal in detecting *ALK* rearrangements. For instance, in the study with 3,244 consecutive NSCLC cases analyzed at two independent French centers, Cabillic et al reported many (55/150) discordant cases between FISH and IHC using the antibody (5A4, 1:50; Abcam, Cambridge, UK) and ultraView Detection Kit (Ventana Medical Systems Inc.).[45]

Overall, several studies have reported 95%–100% sensitivity and specificity of the clone 5A4, in particular the Novocastra/Leica antibody, for *ALK* rearrangement-positive NSCLC with FISH as the gold standard.[8] Subsequently, IHC with the clone 5A4 and iAEP (Histofine ALK iAEP Kit; Nichirei Biosciences Inc., Tokyo, Japan) was approved in Japan as a companion diagnostic test for alectinib.[8] It is important to note, however, that sensitivity and specificity of ALK IHC may differ depending on the cutoff applied when an intensity score or H scoring (opposed to a binary system) is used for the analysis. This issue is elucidated by European Thoracic Oncology Platform Landscape Project that assessed ALK IHC in 1,281 stage I–III adenocarcinomas completely resected at 16 institutions.[46] In the study, the clone 5A4 (no dilution mentioned, Novocastra; Leica Biosystems, Buffalo Gove, IL, USA) with Novolink detection system (Leica Biosystems) was used, and each case was evaluated with both intensity score (0–3+ in any number of cells stained) and H scoring (range: 0–300). When any intensity was considered positive, 6.2% of the study cohort exhibited ALK protein expression. ALK FISH was examined in the ALK IHC positive and matched ALK IHC negative cases (1:2 ratio) and showed that only 35.0% of the samples with any positivity were FISH positive, while the sensitivity of the FISH testing

increased to 81.3% in those with 2+ or 3+ intensity, with the corresponding specificity of 99.0%. In the selected cohort, the positive FISH rates were 0% in those with intensity score 0, 4.2% in intensity score 1+, 60% in intensity score 2+, 90.9% in intensity score 3+; 5.6% in those with H score <120 and 96.2% in H score >120.

## IHC with D5F3

The clone D5F3 recognizes the carboxyl terminus of human ALK protein, and many studies have reported excellent performance of the clone D5F3. Mino-Kenudson et al[38] showed a 100% sensitivity and 99% specificity of IHC with the D5F3 (1:100; Cell Signaling Technology, Danvers, MA, USA) and EnVison+ detection system for *ALK* rearrangement-positive NSCLC diagnosed by FISH. Martinez et al[47] used the clone D5F3 (1:50; Ventana Medical Systems Inc.) combined with ultraView Detection Kit (Ventana Medical Systems Inc.) and reported 83% sensitivity and 100% specificity. Similarly, Minca et al[48] reported 94% sensitivity and 100% specificity of IHC with the D5F3 (1:100) and OptiView Detection Kit (Ventana Medical Systems Inc.). Collectively, several studies on immunohistochemistry with the clone D5F3 (non-CDx) compared with FISH have shown 76%–100% sensitivity and 76%–100% specificity for *ALK* rearrangements in NSCLC (Table 1).[26,38,39,47–66] Relatively low sensitivities that had been reported by some studies were attributed in part to focally/weakly ALK-positive tumors making determination of ALK status challenging; thus, the OptiView Amplification Kit (Ventana Medical Systems Inc.) was applied in conjunction with the OptiView Detection Kit to facilitate assessment of ALK status in focally positive NSCLC specimens. Using the amplification kit, any percentage of strong granular cytoplasmic staining in tumor cells were defined as ALK positive, and a binary scoring algorithm was established.[64] The predictive value of the D5F3 IHC assay with the amplification kit was evaluated on patient samples from the clinical study PROFILE 1004 (a clinical trial testing the efficacy of crizotinib vs standard chemotherapy pemetrexed plus cisplatin or carboplatin in patients with ALK-positive NSCLC). Although its sensitivity and specificity were 86% and 96%, respectively, with *ALK* FISH as the gold standard, the ALK IHC-positive group had a higher response rate and longer PFS compared to the ALK IHC-negative group among *ALK* FISH-positive patients.[64] Subsequently, the ALK (D5F3) CDx assay (the antibody clone D5F3 with OptiView amplification and OptiView detection, Ventana Medical Systems Inc.) coupled to a BenchMark XT or BenchMark ULTRA automated staining instrument (Ventana Medical Systems Inc.) was approved as a companion diagnostic for crizotinib, ceritinib, and/or alectinib in the United States and Japan. More recent studies on D5F3 IHC using a binary scoring algorithm have reported 100% sensitivity and high specificities.[63,65]

Multiple studies have conducted head-to-head comparisons of ALK antibody clones. For example, in an Australian multicenter study, Selinger et al[67] stained NSCLC specimens positive for *ALK* rearrangements confirmed by FISH with three ALK IHC assays: ALK1 (1:50; Dako) with EnVision FLEX+ (Dako), 5A4 (1:25; Leica, Wetzlar, Germany) with ultraView (Ventana Medical Systems Inc.) and amplification, and D5F3 (1:100; Cell Signaling Technology) with OptiView (Ventana Medical Systems Inc.) and amplification. All three assays had 100% sensitivity and 98%–99% specificity. Another multicenter study conducted in Canada compared ALK protein expression using H scoring between clones ALK1 (1:100; Dako), 5A4 (1:30; Novocastra), and D5F3 (1:100; Cell Signaling Technology). In this study, each participating institution used a detection system(s) and an autostainer(s) of its choice, and thus, multiple combinations of antibody clone, detection system, and autostainer were applied. They reported comparative ALK protein expression between the clones 5A4 and D5F3, but generally lower expression by the clone ALK-1 leading to the Pearson correlation between 5A4 vs D5F3 and that between 5A4 vs ALK1 of 0.972 and 0.844, respectively.[68] Other studies also showed high concordance between the antibody clones, but some revealed lower sensitivity of ALK1 compared to D5F3 and 5A4 in detecting *ALK* rearrangements in NSCLC.[8,38,69]

Diagnostic reproducibility on D5F3 IHC between pathologists has also been well established. The study by Wynes et al[57] reported 95% agreement on ALK protein expression by IHC among seven international experts. In this study, 45 *ALK* FISH-positive and 55 *ALK* FISH-negative NSCLC samples were stained with the clone D5F3 (Ventana Medical Systems Inc.) using OptiView Detection Kit and OptiView Amplification Kit on a Benchmark XT autostainer (Ventana Medical Systems Inc.). Similarly, the ALK-Harmonization-Study from Europe using the same D5F3 IHC platform showed high concordance after training of the pathologists.[70] Furthermore, in the aforementioned clinical trial study, between-reader agreement rates involving three independent laboratories exceeded 98%.[64]

While the majority of the above studies used FFPE tissue samples (biopsies and resections), two studies specifically looked at the performance of IHC with the clone D5F3 (Ventana Medical Systems Inc.) on cell blocks from malignant pleural effusion and reported very high concordance with FISH.[43,44] In addition, comparable expression of ALK protein by D5F3 IHC between samples from primary and

**Table 1** Performance of D5F3 immunohistochemistry in detecting *ALK* rearrangements in lung cancer

| Study | N | Country | Histologic types | Scoring system | Sensitivity | Specificity | Note for positivity |
|---|---|---|---|---|---|---|---|
| Mino-Kenudson 2010[38] | 153 | USA | Adenocarcinoma | Binary | 100 | 99.0 | |
| Martinez 2013[47] | 79 | Spain | NSCLC | Binary (cutoff 10%) | 83.3 | 100 | |
| Minca 2013[48] | 249 | USA | NSCLC | Binary | 100 | 100 | Corrected in accordance with the results of the second FISH and clinicopathologic data |
| Ying 2013[49] | 196 | China | Adenocarcinoma | 0–3+ | 100 | 95.0 | >1+ |
| Zhou 2014[58] | 368 | China | Adenocarcinoma | 0–3+ | 100 | 98.8 | >2+, corrected in accordance with the results of RT-PCR |
| Shan 2014[54] | 286 | China | Adenocarcinoma | 0–2+ based on intensity (0–3+) and extent (0–5+) | 100 | 98.8 | >1+, corrected in accordance with the results of RT-PCR |
| Le Quesne 2014[53] | 15 | UK | Adenocarcinoma | Intensity (0–3+) and extent (0–5+) | 100 | 86 | Only FISH-positive cases, intensity ≥1 and extent ≥4 |
| Tantraworasin 2014[55] | 267 | Thailand | NSCLC | Binary | 80 | 94.9 | Only strongly positive staining |
| Demidova 2014[52] | 36 | Russia | NSCLC | 0–3+ | 100 | 100 | >1+ |
| Ali 2014*[50] | 523 | Italy | NSCLC | Binary | 90 | 100 | RT-PCR for *EML4–ALK* variants 1–3 was negative in the two FISH+/IHC− cases |
| Conde 2014[51] | 103 | Spain | NSCLC | 0–3+ | 98 | 100 | >2+ |
| Wang 2014*[56] | 430 | China | Adenocarcinoma | Binary | 100 | 98.2 | |
| Wynes 2015[57] | 103 | USA | NSCLC | Binary | 90 | 95 | |
| Pekar-Zlotin 2015[60] | 51 | Israel | Adenocarcinoma | 0–3+ | 100 | 97.7 | H score ≥40, corrected in accordance with the results of NGS |
| Rogers 2015[61] | 362 | Australia | NSCLC | Intensity (0–3+) and extent (0–3+) | 100 | 99.7 | |
| Lantuejoul 2015[59] | 547 | France | Adenocarcinoma | 0–3+ | 89 | 76 | ≥10% of the cells with a 1–3+ intensity, corrected in accordance with the results of RT-PCR |
| Savic 2015[62] | 72 | Switzerland | NSCLC | 0–3+ | 96 | 100 | >3+ |
| Ilie 2015*[26] | 176 | France | Adenocarcinoma | Binary | 81 | 99 | The five FISH+/IHC− cases were FISH borderline positive (15%–20%); three overexpressed c-MET and responded to crizotinib, and two without c-MET expression progressed on crizotinib |
| Wang 2016[39] | 595 | China | Adenocarcinoma | 0–3+ | 75.9 | 99.8 | >1+ |
| Thorne-Nuzzo 2017*[64] | 933 | Global (clinical trial) | NSCLC | Binary | 86.0 | 96.3 | Overall response rate to crizotinib: 86.7% for FISH+/IHC+ and 33.3% for FISH+/IHC− cases ($P$=0.0083) |
| Wagle 2017*[65] | 200 | India | Adenocarcinoma | Binary | 100 | 90.5 | |
| Murthy 2017*[63] | 341 | India | Adenocarcinoma | Binary | 100 | 94.4 | |
| Kheng 2018[66] | 304 | UK | NSCLC | Binary | 100 | 96.6 | |

**Note:** *Studies in which the ALK (D5F3) CDx immunohistochemistry assay or an equivalent assay was used in conjunction with a binary scoring algorithm.

**Abbreviations:** ALK, anaplastic lymphoma kinase; FISH, fluorescent in situ hybridization; IHC, immunohistochemistry; NSCLC, non-small-cell lung cancer; RT-PCR, real-time polymerase chain reaction.

metastatic sites has also been well documented with concordance rates of 94%–100%.[71–73]

## Discordant IHC and FISH results

Several studies have compared the performance of IHC with the clone D5F3 vs FISH in detecting *ALK*-rearranged NSCLCs. Perlar-Zlotin et al reported the sensitivity and specificity of 100% and 97.7%, respectively, for D5F3 IHC and 42.9% and 97.7%, respectively, for FISH with NGS as the gold standard in 51 lung adenocarcinoma patients.[60] More recently, van der Wekken et al looked at the response to crizotinib in 29 stage IV NSCLC patients whose tumors had been shown to have *ALK* rearrangements by FISH and/or the ALK (D5F3) CDx assay, and reported that all immunohistochemistry-positive (IHC+) patients responded to crizotinib except for three with primary resistance, while no tumor response was observed in 13 FISH-positive (FISH+) but immunohistochemistry-negative (IHC−) patients.[74] The results were confirmed in an external cohort of 16 patients. These results are in line with those of the clinical trial study.[64] Overall, IHC+/FISH− cases are considered ALK+ and will likely benefit from treatment with crizotinib.[75] While the vast majority of IHC−/FISH borderline+ results are attributed to the technical/interpretation difficulty of ALK FISH,[28] and are considered ALK−,[75] some IHC−/FISH borderline+ results have been reported in NSCLCs with *MET* overexpression that responded to crizotinib (a MET and ALK inhibitor).[26] IHC−/FISH clearly+ results are rare and may be fixation artifacts,[31] or there may be no transcription of the *ALK* fusion gene.[75] However, additional NGS-based or treatment response-based clinical observation studies are warranted to formulate a clinically meaningful statement on these rare events.[75]

## ALK IHC as a standalone test for *ALK* rearrangements in NSCLC

As discussed above, several lines of evidence support the notion that IHC with the clone D5F3, in particular the ALK (D5F3) CDx assay, can be used as a standalone test to select advanced NSCLC patients for treatment with ALK TKIs. Subsequently, the recently updated molecular testing guideline (put forth by the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology) has designated properly validated IHC assays for ALK as an equivalent alternative to *ALK* FISH.[40,76–78] Tissue samples with equivocal results should be tested and confirmed by other methods (FISH, RT-PCR, and/or NGS), however. From a clinical perspective, a recent clinical trial for alectinib required *ALK* rearrangements confirmed by IHC with the ALK (D5F3) CDx assay,[18] while previous clinical trials for crizotinib required *ALK* FISH positivity.[14,15]

## Pitfalls of ALK IHC

Several pitfalls of ALK IHC, including that with the clone D5F3, should be noted. First, signet ring cells or tumor cells with cytoplasmic mucin, often seen in *ALK* rearrangement-positive NSCLCs, may be a source of false negative results due to the limited expression in thin and scanty cytoplasm. Therefore, tissue samples with mucin-containing tumor cells require careful interpretation of ALK IHC. Second, false positive staining may be seen in alveolar macrophages, nerve, gangiloin cells, airway epithelial cells, extracellular mucin, and necrotic debris, particularly when strong IHC amplification systems are used.[68] False positive cytoplasmic staining in NSCLC, albeit often weaker than true positive expression, has also been identified in association with the clone D5F3 and tyramide amplification system. Third, tumor cells with neuroendocrine differentiation (small cell carcinoma, large cell neuroendocrine carcinoma, and carcinoid tumor) have been reported to show false positive reactivity to ALK IHC,[24,79,80] although their expressions are typically heterogeneous or in a checkerboard pattern. Fourth, quality control of staining was found to be important. A study of international quality assessment involving 30 countries showed that about 10% of the slides stained with D5F3 IHC were judged as unacceptable or borderline in quality by pathologists.[81] Furthermore, NSCLCs with *KIF5B-ALK* rearrangements have been reported to show dot-like staining by ALK IHC.[6] Thus, it is important to evaluate/confirm samples exhibiting focal and/or equivocal expressions with *ALK* FISH, RT-PCR, and/or NGS.

## Summary

IHC with the ALK antibody clone D5F3, in particular the ALK (D5F3) CDx assay, has been proven to have great sensitivity and specificity for *ALK* rearrangements in NSCLC, and can be used as a standalone test in practice. Nevertheless, it is important to understand several potential pitfalls of ALK IHC and further evaluate specimens exhibiting focal/equivocal expressions with other ALK testing methods.

## Disclosure

M Mino-Kenudson serves as a consultant for Merrimack Pharmaceuticals and H3 Biomedicine. The authors report no other conflicts of interest with this work.

## References


1. Barlesi F, Mazieres J, Merlio JP, et al. Routine molecular profiling of patients with advanced non-small-cell lung cancer: results of a 1-year nationwide programme of the French Cooperative Thoracic Intergroup (IFCT). *Lancet*. 2016;387(10026):1415–1426.
2. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004;350(21):2129–2139.
3. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature*. 2007;448(7153):561–566.
4. Horn L, Pao W. EML4-ALK: honing in on a new target in non-small-cell lung cancer. *J Clin Oncol*. 2009;27(26):4232–4235.
5. Pao W, Girard N. New driver mutations in non-small-cell lung cancer. *Lancet Oncol*. 2011;12(2):175–180.
6. Takeuchi K, Choi YL, Togashi Y, et al. KIF5B-ALK, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. *Clin Cancer Res*. 2009;15(9):3143–3149.
7. Rosenbaum JN, Bloom R, Forys JT, et al. Genomic heterogeneity of ALK fusion breakpoints in non-small-cell lung cancer. *Mod Pathol*. 2018;31(5):791–808.
8. Tsao MS, Hirsh FR, Yatabe Y, editors. *IASLC Atlas of ALK and ROS1 Testing in Lung Cancer*. 2nd ed. Fort Meyers: Editorial Rx Press; 2016.
9. Koivunen JP, Mermel C, Zejnullahu K, et al. EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. *Clin Cancer Res*. 2008;14(13):4275–4283.
10. Inamura K, Takeuchi K, Togashi Y, et al. EML4-ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset. *Mod Pathol*. 2009;22(4):508–515.
11. Wong DW, Leung EL, So KK, et al. The EML4-ALK fusion gene is involved in various histologic types of lung cancers from nonsmokers with wild-type EGFR and KRAS. *Cancer*. 2009;115(8):1723–1733.
12. Shaw AT, Yeap BY, Mino-Kenudson M, et al. Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. *J Clin Oncol*. 2009;27(26):4247–4253.
13. Inamura K, Takeuchi K, Togashi Y, et al. EML4-ALK fusion is linked to histological characteristics in a subset of lung cancers. *J Thorac Oncol*. 2008;3(1):13–17.
14. Shaw AT, Kim DW, Nakagawa K, et al. Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. *N Engl J Med*. 2013;368(25):2385–2394.
15. Solomon BJ, Mok T, Kim DW, et al. First-line crizotinib versus chemotherapy in ALK-positive lung cancer. *N Engl J Med*. 2014;371(23):2167–2177.
16. Yoshida T, Oya Y, Tanaka K, et al. Differential crizotinib response duration among ALK fusion variants in ALK-positive non-small-cell lung cancer. *J Clin Oncol*. 2016;34(28):3383–3389.
17. Hida T, Nokihara H, Kondo M, et al. Alectinib versus crizotinib in patients with ALK-positive non-small-cell lung cancer (J-ALEX): an open-label, randomised phase 3 trial. *Lancet*. 2017;390(10089):29–39.
18. Peters S, Camidge DR, Shaw AT, et al. Alectinib versus crizotinib in untreated ALK-positive non-small-cell lung cancer. *N Engl J Med*. 2017;377(9):829–838.
19. Soria JC, Tan DSW, Chiari R, et al. First-line ceritinib versus platinum-based chemotherapy in advanced ALK-rearranged non-small-cell lung cancer (ASCEND-4): a randomised, open-label, phase 3 study. *Lancet*. 2017;389(10072):917–929.
20. Shaw AT, Kim TM, Crinò L, et al. Ceritinib versus chemotherapy in patients with ALK-rearranged non-small-cell lung cancer previously given chemotherapy and crizotinib (ASCEND-5): a randomised, controlled, open-label, phase 3 trial. *Lancet Oncol*. 2017;18(7):874–886.
21. Shaw AT, Felip E, Bauer TM, et al. Lorlatinib in non-small-cell lung cancer with ALK or ROS1 rearrangement: an international, multicentre, open-label, single-arm first-in-man phase 1 trial. *Lancet Oncol*. 2017;18(12):1590–1599.
22. Camidge DR, Kono SA, Flacco A, et al. Optimizing the detection of lung cancer patients harboring anaplastic lymphoma kinase (ALK) gene rearrangements potentially suitable for ALK inhibitor treatment. *Clin Cancer Res*. 2010;16(22):5581–5590.
23. Yoshida A, Tsuta K, Watanabe S, et al. Frequent ALK rearrangement and TTF-1/p63 co-expression in lung adenocarcinoma with signet-ring cell component. *Lung Cancer*. 2011;72(3):309–315.
24. Murakami Y, Mitsudomi T, Yatabe Y. A screening method for the ALK fusion gene in NSCLC. *Front Oncol*. 2012;2:24.
25. Camidge DR, Skokan M, Kiatsimkul P, et al. Native and rearranged ALK copy number and rearranged cell count in non-small cell lung cancer: implications for ALK inhibitor therapy. *Cancer*. 2013;119(22):3968–3975.
26. Ilie MI, Bence C, Hofman V, et al. Discrepancies between FISH and immunohistochemistry for assessment of the ALK status are associated with ALK 'borderline'-positive rearrangements or a high copy number: a potential major issue for anti-ALK therapeutic strategies. *Ann Oncol*. 2015;26(1):238–244.
27. Selinger C, Cooper W, Lum T, et al. Equivocal ALK fluorescence in-situ hybridization (FISH) cases may benefit from ancillary ALK FISH probe testing. *Histopathology*. 2015;67(5):654–663.
28. von Laffert M, Stenzinger A, Hummel M, et al. ALK-FISH borderline cases in non-small cell lung cancer: implications for diagnostics and clinical decision making. *Lung Cancer*. 2015;90(3):465–471.
29. von Laffert M, Warth A, Penzel R, et al. Anaplastic lymphoma kinase (ALK) gene rearrangement in non-small cell lung cancer (NSCLC): results of a multi-centre ALK-testing. *Lung Cancer*. 2013;81(2):200–206.
30. Marchetti A, Barberis M, Papotti M, et al. ALK rearrangement testing by FISH analysis in non-small-cell lung cancer patients: results of the first italian external quality assurance scheme. *J Thorac Oncol*. 2014;9(10):1470–1476.
31. Abe H, Kawahara A, Azuma K, et al. Heterogeneity of anaplastic lymphoma kinase gene rearrangement in non-small-cell lung carcinomas: a comparative study between small biopsy and excision samples. *J Thorac Oncol*. 2015;10(5):800–805.
32. Takeuchi K, Choi YL, Soda M, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. *Clin Cancer Res*. 2008;14(20):6618–6624.
33. Letovanec I, Finn S, Zygoura P, et al. Evaluation of NGS and RT-PCR methods for ALK rearrangement in European NSCLC patients: results from the European Thoracic Oncology Platform Lungscape Project. *J Thorac Oncol*. 2018;13(3):413–425.
34. Drilon A, Wang L, Arcila ME, et al. Broad, hybrid capture-based next-generation sequencing identifies actionable genomic alterations in lung adenocarcinomas otherwise negative for such alterations by other genomic testing approaches. *Clin Cancer Res*. 2015;21(16):3631–3639.
35. D'Haene N, Fontanges Q, de Nève N, et al. Clinical application of targeted next-generation sequencing for colorectal cancer patients: a multicentric Belgian experience. *Oncotarget*. 2018;9(29):20761–20768.
36. Oliveira DM, Mirante T, Mignogna C, et al. Simultaneous identification of clinically relevant single nucleotide variants, copy number alterations and gene fusions in solid tumors by targeted next-generation sequencing. *Oncotarget*. 2018;9(32):22749–22768.
37. Rodig SJ, Mino-Kenudson M, Dacic S, et al. Unique clinicopathologic features characterize ALK-rearranged lung adenocarcinoma in the western population. *Clin Cancer Res*. 2009;15(16):5216–5223.
38. Mino-Kenudson M, Chirieac LR, Law K, et al. A novel, highly sensitive antibody allows for the routine detection of ALK-rearranged lung adenocarcinomas by standard immunohistochemistry. *Clin Cancer Res*. 2010;16(5):1561–1571.
39. Wang Q, Zhao L, Yang X, et al. Antibody 1A4 with routine immunohistochemistry demonstrates high sensitivity for ALK rearrangement screening of Chinese lung adenocarcinoma patients: a single-center large-scale study. *Lung Cancer*. 2016;95:39–43.

40. Lindeman NI, Cagle PT, Aisner DL, et al. Updated molecular testing guideline for the selection of lung cancer patients for treatment with targeted tyrosine kinase inhibitors: guideline from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology. *Arch Pathol Lab Med*. 2018;142(3):321–346.
41. Thunnissen E, Allen TC, Adam J, et al. Immunohistochemistry of pulmonary biomarkers: a perspective from members of the Pulmonary Pathology Society. *Arch Pathol Lab Med*. 2018;142(3):408–419.
42. Paik JH, Choe G, Kim H, et al. Screening of anaplastic lymphoma kinase rearrangement by immunohistochemistry in non-small cell lung cancer: correlation with fluorescence in situ hybridization. *J Thorac Oncol*. 2011;6(3):466–472.
43. Kim H, Yoo SB, Choe JY, et al. Detection of ALK gene rearrangement in non-small cell lung cancer: a comparison of fluorescence in situ hybridization and chromogenic in situ hybridization with correlation of ALK protein expression. *J Thorac Oncol*. 2011;6(8):1359–1366.
44. Jokoji R, Yamasaki T, Minami S, et al. Combination of morphological feature analysis and immunohistochemistry is useful for screening of EML4-ALK-positive lung adenocarcinoma. *J Clin Pathol*. 2010;63(12):1066–1070.
45. Cabillic F, Gros A, Dugay F, et al. Parallel FISH and immunohistochemical studies of ALK status in 3244 non-small-cell lung cancers reveal major discordances. *J Thorac Oncol*. 2014;9(3):295–306.
46. Blackhall FH, Peters S, Bubendorf L, et al. Prevalence and clinical outcomes for patients with ALK-positive resected stage I to III adenocarcinoma: results from the European Thoracic Oncology Platform Lungscape Project. *J Clin Oncol*. 2014;32(25):2780–2787.
47. Martinez P, Hernández-Losa J, Montero MÁ, et al. Fluorescence in situ hybridization and immunohistochemistry as diagnostic methods for ALK positive non-small cell lung cancer patients. *PLoS One*. 2013;8(1):e52261.
48. Minca EC, Portier BP, Wang Z, et al. ALK status testing in non-small cell lung carcinoma: correlation between ultrasensitive IHC and FISH. *J Mol Diagn*. 2013;15(3):341–346.
49. Ying J, Guo L, Qiu T, et al. Diagnostic value of a novel fully automated immunochemistry assay for detection of ALK rearrangement in primary lung adenocarcinoma. *Ann Oncol*. 2013;24(10):2589–2593.
50. Alì G, Proietti A, Pelliccioni S, et al. ALK rearrangement in a large series of consecutive non-small cell lung cancers: comparison between a new immunohistochemical approach and fluorescence in situ hybridization for the screening of patients eligible for crizotinib treatment. *Arch Pathol Lab Med*. 2014;138(11):1449–1458.
51. Conde E, Suárez-Gauthier A, Benito A, et al. Accurate identification of ALK positive lung carcinoma patients: novel FDA-cleared automated fluorescence in situ hybridization scanning system and ultrasensitive immunohistochemistry. *PLoS One*. 2014;9(9):e107200.
52. Demidova I, Barinov A, Savelov N, et al. Immunohistochemistry, fluorescence in situ hybridization, and reverse transcription-polymerase chain reaction for the detection of anaplastic lymphoma kinase gene rearrangements in patients with non-small cell lung cancer: potential advantages and methodologic pitfalls. *Arch Pathol Lab Med*. 2014;138(6):794–802.
53. Le Quesne J, Maurya M, Yancheva SG, et al. A comparison of immunohistochemical assays and FISH in detecting the ALK translocation in diagnostic histological and cytological lung tumor material. *J Thorac Oncol*. 2014;9(6):769–774.
54. Shan L, Lian F, Guo L, Yang X, Ying J, Lin D. Combination of conventional immunohistochemistry and qRT-PCR to detect ALK rearrangement. *Diagn Pathol*. 2014;9:3.
55. Tantraworasin A, Lertprasertsuke N, Kongkarnka S, et al. Retrospective study of ALK rearrangement and clinicopathological implications in completely resected non-small cell lung cancer patients in Northern Thailand: role of screening with D5F3 antibodies. *Asian Pac J Cancer Prev*. 2014;15(7):3057–3063.
56. Wang Y, Wang S, Xu S, Qu J, Liu B. Clinicopathologic features of patients with non-small cell lung cancer harboring the EML4-ALK fusion gene: a meta-analysis. *PLoS One*. 2014;9(10):e110617.
57. Wynes MW, Sholl LM, Dietel M, et al. An international interpretation study using the ALK IHC antibody D5F3 and a sensitive detection kit demonstrates high concordance between ALK IHC and ALK FISH and between evaluators. *J Thorac Oncol*. 2014;9(5):631–638.
58. Zhou J, Zhao J, Sun K, et al. Accurate and economical detection of ALK positive lung adenocarcinoma with semiquantitative immunohistochemical screening. *PLoS One*. 2014;9(3):e92828.
59. Lantuejoul S, Rouquette I, Blons H, et al. French multicentric validation of ALK rearrangement diagnostic in 547 lung adenocarcinomas. *Eur Respir J*. 2015;46(1):207–218.
60. Pekar-Zlotin M, Hirsch FR, Soussan-Gutman L, et al. Fluorescence in situ hybridization, immunohistochemistry, and next-generation sequencing for detection of EML4-ALK rearrangement in lung cancer. *Oncologist*. 2015;20(3):316–322.
61. Rogers TM, Russell PA, Wright G, et al. Comparison of methods in the detection of ALK and ROS1 rearrangements in lung cancer. *J Thorac Oncol*. 2015;10(4):611–618.
62. Savic S, Diebold J, Zimmermann AK, et al. Screening for ALK in non-small cell lung carcinomas: 5A4 and D5F3 antibodies perform equally well, but combined use with FISH is recommended. *Lung Cancer*. 2015;89(2):104–109.
63. Murthy SS, Rajappa SJ, Gundimeda SD, et al. Anaplastic lymphoma kinase status in lung cancers: an immunohistochemistry and fluorescence *in situ* hybridization study from a tertiary cancer center in India. *Indian J Cancer*. 2017;54(1):231–235.
64. Thorne-Nuzzo T, Williams C, Catallini A, et al. A sensitive ALK immunohistochemistry companion diagnostic test identifies patients eligible for treatment with crizotinib. *J Thorac Oncol*. 2017;12(5):804–813.
65. Wagle PB, Jambhekar NA, Kumar R, et al. A comparative analysis of immunohistochemistry and fluorescent *in situ* hybridization assay to detect anaplastic lymphoma kinase status in lung adenocarcinoma cases: a search for a testing algorithm. *Indian J Cancer*. 2017;54(1):148–154.
66. Kheng YC, Walsh K, Williams L, Wallace WA, Harrison DJ, Oniscu A. ALK immunohistochemistry is highly sensitive and specific for the detection of ALK translocated lung adenocarcinomas: lessons from an audit of lung cancer molecular testing. *J R Coll Physicians Edinb*. 2018;48(1):20–24.
67. Selinger CI, Rogers TM, Russell PA, et al. Testing for ALK rearrangement in lung adenocarcinoma: a multicenter comparison of immunohistochemistry and fluorescent in situ hybridization. *Mod Pathol*. 2013;26(12):1545–1553.
68. Cutz JC, Craddock KJ, Torlakovic E, et al. Canadian anaplastic lymphoma kinase study: a model for multicenter standardization and optimization of ALK testing in lung cancer. *J Thorac Oncol*. 2014;9(9):1255–1263.
69. Conklin CM, Craddock KJ, Have C, et al. Immunohistochemistry is a reliable screening tool for identification of ALK rearrangement in non-small-cell lung carcinoma and is antibody dependent. *J Thorac Oncol*. 2013;8(1):45–51.
70. von Laffert M, Warth A, Penzel R, et al. Multicenter immunohistochemical ALK-testing of non-small-cell lung cancer shows high concordance after harmonization of techniques and interpretation criteria. *J Thorac Oncol*. 2014;9(11):1685–1692.
71. Hou L, Ren S, Su B, et al. High concordance of ALK rearrangement between primary tumor and paired metastatic lymph node in patients with lung adenocarcinoma. *J Thorac Dis*. 2016;8(6):1103–1111.
72. Ma W, Guo L, Shan L, Liu X, Lyu N, Ying J. Homogeneity and high concordance of ALK translocation in primary lung adenocarcinoma and paired lymph node metastasis. *Sci Rep*. 2017;7(1):10961.
73. Trejo Bittar HE, Luvison A, Miller C, Dacic S. A comparison of ALK gene rearrangement and ALK protein expression in primary lung carcinoma and matched metastasis. *Histopathology*. 2017;71(2):269–277.
74. van der Wekken AJ, Pelgrim R, 't Hart N, et al. Dichotomous ALK-IHC is a better predictor for ALK inhibition outcome than traditional ALK-FISH in advanced non-small cell lung cancer. *Clin Cancer Res*. 2017;23(15):4251–4258.

75. von Laffert M, Schirmacher P, Warth A, et al. ALK-testing in non-small cell lung cancer (NSCLC): immunohistochemistry (IHC) and/or fluorescence in-situ hybridisation (FISH)?: statement of the Germany Society for Pathology (DGP) and the Working Group Thoracic Oncology (AIO) of the German Cancer Society e.V. (Stellungnahme der Deutschen Gesellschaft für Pathologie und der AG Thorakale Onkologie der Arbeitsgemeinschaft Onkologie/Deutsche Krebsgesellschaft e.V.. *Lung Cancer*. 2017;103:1–5.
76. Mino-Kenudson M. Immunohistochemistry in anaplastic lymphoma kinase and proto-oncogene tyrosine-protein kinase ROS. *Arch Pathol Lab Med*. 2018;142(7):792–793.
77. Lindeman NI, Cagle PT, Aisner DL, et al. Updated molecular testing guideline for the selection of lung cancer patients for treatment with targeted tyrosine kinase inhibitors: guideline from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology. *J Mol Diagn*. 2018;20(2):129–159.
78. Lindeman NI, Cagle PT, Aisner DL, et al. Updated molecular testing guideline for the selection of lung cancer patients for treatment with targeted tyrosine kinase inhibitors: guideline from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology. *J Thorac Oncol*. 2018;13(3):323–358.
79. Takeuchi K. Interpretation of anti-ALK immunohistochemistry results. *J Thorac Oncol*. 2013;8(7):e67–e68.
80. Nakamura H, Tsuta K, Yoshida A, et al. Aberrant anaplastic lymphoma kinase expression in high-grade pulmonary neuroendocrine carcinoma. *J Clin Pathol*. 2013;66(8):705–707.
81. Ibrahim M, Parry S, Wilkinson D, et al. ALK immunohistochemistry in NSCLC: discordant staining can impact patient treatment regimen. *J Thorac Oncol*. 2016;11(12):2241–2247.