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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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.

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