Comparing four different ALK antibodies with manual immunohistochemistry (IHC) to screen for ALK-rearranged non-small cell lung cancer (NSCLC)

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**ABSTRACT**

Objectives: Anaplastic lymphoma kinase (ALK)-rearranged non-small cell lung cancer (NSCLC) screening is essential to its treatment such as crizotinib. Different assays have been developed to detect ALK rearrangements, such as fluorescence in situ hybridization (FISH), reverse transcriptase-PCR (RT-PCR), and immunohistochemistry (IHC). However, ALK detection has not been applied widely in all hospitals. Moreover, IHC has been proposed to be a pre-screening tool because of its wide application in clinics. Since the low expression of ALK protein, the sensitivity and specificity of ALK antibody are the keys to the success of IHC screening. Therefore, we compared different antibodies to find the best one for IHC detection.

Materials and methods: We evaluated ALK expression by four different ALK antibodies: clone D5F3 (Ventana), clone D5F3 (CST), clone 1A4/1H7 (OriGene Tech.), and clone 5A4 (Abcam) based on manual IHC in a cohort of 60 NSCLCs. The results were compared with those from automated IHC (clone D5F3, Ventana). All cases were evaluated independently by ALK FISH.

Results: 32 ALK-positive and 28 ALK-negative NSCLCs were identified by automated IHC (D5F3, Ventana) and FISH analysis. Based on conventional manual IHC, the sensitivity of four antibodies—D5F3 (Ventana), D5F3 (CST), 1A4/1H7 (OriGene Tech.), and 5A4 (Abcam)—was 93.8%, 84.4%, 93.8%, and 56.3%, respectively. Their specificities and positive predictive values were 100%. The percentage of strong-moderate staining was 65.6%, 62.5%, 68.8%, and 21.9%, respectively. Compared with automated IHC (D5F3, Ventana), each staining concordance was 96.7%, 91.7%, 96.7%, and 76.7%, respectively, and each presented staining heterogeneity (weak–moderate–strong intensity).

Conclusion: These data indicated that manual IHC with a more reliable ALK antibody might provide an effective strategy for screening ALK gene rearrangements in all NSCLC patients, followed by confirmatory FISH analysis in IHC-positive cases.

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1. Introduction

Translocation of anaplastic lymphoma kinase gene (ALK) and echinoderm microtubule-associated protein-like 4 (EML4) in lung adenocarcinoma was first reported by Soda et al. [1]. In NSCLC, the well-known variation of ALK gene rearrangement is EML4-ALK, and other translocation events such as TFG-ALK, KIF5B-ALK, and KLC1-ALK also have been found. The rearrangements subsequently lead to constitutive activation of the ALK tyrosine kinase. ALK-rearranged NSCLC has been shown to be exquisitely sensitive to therapy with ALK tyrosine inhibitors (crizotinib), with an overall response rate of 57% and a 72% progression-free survival of 6 months or greater [2,3]. ALK molecular detection has become crucial for selecting the right patients for target therapies in clinical practice [4,5]. The methods for detecting ALK rearrangements include fluorescence in situ hybridization (FISH), reverse transcription-PCR (RT-PCR), and automated immunohistochemistry (IHC) [6,7]. However, RT-PCR is not recommended as a first-line diagnostic method for diagnosing ALK-rearranged NSCLCs owning to its higher failure rate and the risk of false negatives [8]. FISH has been shown to be both sensitive and specific. However, it requires specialized technical resources and expertise and is expensive and time-consuming; as a result,
the assay is not readily available in all laboratories [9,10]. IHC screening is relatively economical and can be performed routinely in most diagnostic laboratories. Minca et al. and Ying et al. used ultrasensitive automated IHC (clone D5F3, Ventana) and revealed that it has a very high correlation with FISH in assessing ALK status [11,12]. Unfortunately, the automated IHC apparatus is not used as a population-wide screening tool, such as are currently only available in larger hospitals of China. Therefore, biomarker screening appears to be underutilized in routine care. Recently, some studies about ALK protein expression based on manual IHC were reported, but it is not cleared which antibody is best suited for this application [13–15]. To develop a potential screening method with a reliable ALK antibody based on manual IHC to detect ALK-rearranged NSCLCs, we evaluated ALK protein expression by different ALK antibodies with manual and automated IHC in NSCLCs. The aim was to select a wider and more economical assay for clinical ALK-detecting practice.

2. Materials and methods

2.1. NSCLC specimens

All ALK-rearranged NSCLC cases from Nanjing Jinling Hospital in 2013 and the same number of cases of ALK-negative NSCLCs were retrospectively collected. After removing cases that had few or no tumor cells, 60 formalin-fixed paraffin-embedded (FFPE) samples of NSCLC were selected, including biopsy specimens (percutaneous lung biopsy and transbronchial biopsy) and excision specimens (primary and metastatic mass). In the 60 NSCLCs, 38 were from male patients and 20 were from female patients, with an average age of 54.3 (range: 30–82). 22 patients were in stage I and II, and 38 cases were in stage III and IV. The tumor size was from 0.9 cm to 12 cm (average 3.8 cm). The samples were obtained from 16 biopsy specimens of primary lung cancers, 40 lung lobectomies, and 4 excision specimens of metastatic mass (from 2 supravacular lymph nodes, 1 frontal lobe, and 1 parietal lobe). Patients had never been treated with either chemotherapy or radiotherapy before the specimens were obtained. The biopsies and excision specimens were sectioned to 4 μm thick in preparation for Haematoxylin and Eosin (H&E) staining, IHC, and FISH.

2.2. Immunohistochemistry (IHC)

60 cases were conducted by anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody (Ventana, Tucson, AZ) immunohistochemical staining with the BenchMark XT automated slide processing system (BenchMark, Ventana, Tucson, AZ) and additional OptiView enhanced detection and amplification system, according to the manufacturer’s protocol. In parallel with this, we treated each slide with negative and positive controls. The staining was evaluated using a binary system [7,8]: exclusively stained cases with strong granular cytoplasmic staining in any percentage of tumor cells was diagnosed as a positive result, and the absence of strong (3+) granular cytoplasmic staining was diagnosed as a negative result. 60 cases were independently evaluated with standardized manual IHC detection systems. Antigen retrieval was performed using a pressure cooker with citrate buffer (pH 6.0) for 3 min. Sections were then incubated with ALK antibody in the humid chambers for 4 h at RT, and with EnVision detection kit (Dako) for 30 min at RT without signal amplification system. The four different ALK antibodies are as follows: (1) anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody (Ventana, Tucson, AZ); (2) anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody (Cell Signaling Technology, Billerica, MA: 1:100); (3) anti-ALK (1A4/1H7) Mouse Monoclonal Primary Antibody (OriGene Tech, USA); and (4) anti-ALK (5A4) Mouse Monoclonal Primary Antibody (Abcam, UK: 1:50). ALK IHC was evaluated by a modified semiquantitative graded criteria based on a scoring system as follows [8]: An IHC score of 3+ was given for strong and granular cytoplasmic staining staining in most of the tumor cells. At least more than 75% of them have diffusely homogeneity in distribution. A score 2+ was given for moderate and smooth cytoplasmic staining (which also can partly present strong staining) in more than 50% of the tumor cells. A score of 1+ was given for faint and focal cytoplasmic staining with less than score 2+ criteria. And a score of 0 was given for completely no staining. IHC scoring was performed by three pathologists without foreknowledge of the FISH results. All ALK-rearranged cases contained wild-type EGFR and KRAS.

2.3. Fluorescence in situ hybridization (FISH)

FISH was carried out for all cases. ALK break-apart probe (Vysis LSI ALK; Abbott Molecular, Abbott Park, IL) was used to evaluate the ALK genetic fusion status by FISH according to the manufacturer’s technical instructions and interpretation standard. The 3’ and 5’ end of ALK gene was labeled with red and green fluorescence respectively. A minimum of 100 cancer cells were observed by fluorescent microscopy. Wild-type ALK would have a yellow signal (red–green fusion). If >15% of cancer cells displayed the followed signals, they would be considered as ALK-rearranged: (1) red and green signals were separated by ≥2 signal diameters; (2) a deleted 5’ ALK green signal (solitary red signal) was observed in tumor cell nuclei [8,14]. H&E and FISH slides for all cases were reviewed by two pathologists to confirm that scoring was carried out in the tumor cell population.

3. Results

Using the automated IHC (clone D5F3, Ventana), 32 specimens showed positive staining (Fig. 1A) and 28 cases presented negative staining. They were confirmed as 32 ALK-rearranged NSCLCs and 28 negative cases by FISH in a double-blind study (Fig. 1B). The cases with IHC-positive/FISH-negative or IHC-negative/FISH-positive were excluded from the statistical analysis. In the 32 IHC-positive specimens, 29 (29/32, 90.6%) cases displayed positive staining in almost all tumor cells, and 3 (3/32, 9.4%) had partial (70–90%) staining. 24 (24/32, 75%) IHC-positive specimens revealed homogeneous strong staining, and 8 (8/32, 25%) specimens exhibited (from moderate to strong) heterogeneous staining. Four antibodies of three clones from four different manufacturers—D5F3 (Ventana), D5F3 (CST), 1A4/1H7 (OriGene Tech.), and 5A4 (Abcam)—were applied on 60 NSCLCs in combination with manual IHC (no amplification). Each slide was evaluated as IHC score 3+, score 2+, score 1+, or score 0. The further analysis was in Tables 1 and 2. None of the 28 no-ALK-rearranged NSCLCs demonstrated any staining with four different antibodies. Among the 32 ALK-rearranged NSCLCs, 30 (93.8%, 30/32) cases showed positive staining with clone D5F3 (Ventana), 27 (84.4%, 27/32) displayed positive staining with clone D5F3 (CST), 30 (93.8%, 30/32) showed positive staining with clone 1A4/1H7, and 18 (56.3%, 18/32) showed positive staining with 5A4 (Abcam). The percentage of strong/medium-staining was 65.6%, 62.5%, 68.8%, and 21.9%, respectively. The specificities and positive predictive values from four antibodies were 100%. Among the four antibodies used, the highest sensitivity was 93.8% from D5F3 (Ventana) and 1A4/1H7 (OriGene Tech.), and the negative predictive value reached up to 93.3% from D5F3 (Ventana) and 1A4/1H7 (OriGene Tech.). ALK protein expression was observed in tumor cells with a predominantly cytoplasmic staining without nonspecific or background staining. (Figs. 2A–D and 3B–D).
Table 1
ALK protein expression in NSCLCs based on manual IHC with four different antibodies (n=60).

<table>
<thead>
<tr>
<th>Antibody (Clone)</th>
<th>Rearranged +</th>
<th>Rearranged −</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone D5F3 (Ventana)</td>
<td>25%</td>
<td>0</td>
</tr>
<tr>
<td>Clone D5F3 (CST)</td>
<td>25%</td>
<td>0</td>
</tr>
<tr>
<td>Clone 1A4/1H7 (OriGene)</td>
<td>18.8%</td>
<td>0</td>
</tr>
<tr>
<td>Clone 5A4 (Abcam)</td>
<td>9.4%</td>
<td>0</td>
</tr>
</tbody>
</table>

IHC = immunohistochemistry; NSCLC = non-small cell lung carcinoma.
Rearranged +: ALK-rearranged NSCLC by FISH (fluorescence in situ hybridization) testing; rearranged −: no-ALK-rearranged NSCLC by FISH testing.

When the results from automated IHC (clone D5F3, Ventana) were used as the standard reference, the expression concordance between the standard and clone D5F3 (Ventana), clone D5F3 (CST), clone 1A4/1H7, and 5A4 (Abcam) was 96.7%, 91.7%, 96.7%, and 76.7%, respectively. The percentage of positive cells declined as low as 30%. The staining intensity decreased obviously, and the heterogeneity (weak–moderate–strong intensity) increased (40.6%) compared with automated IHC (clone D5F3, Ventana) (Fig. 2B–D vs Fig. 3B and C).

3.1. Comparison on staining results among cases from different ways of sampling

Among 32 ALK-rearranged NSCLCs, the sensitivity in 20 excision cases with four antibodies was 95%, 95%, 95%, and 65%. In 12 biopsy specimens, the sensitivity was 91.7%, 66.7%, 91.7%, and 41.7%. The expression in excision samples was superior to those of biopsy specimens. The staining intensity (2+ to 3+) of excision samples was 75%, 75%, 90%, and 30%, but from the biopsy specimens it was 30%, 40%, 55%, and 25% (Fig. 2B–D vs Fig. 3B and C). In addition, 4 metastatic lung adenocarcinomas (from 2 supraclavicular lymph nodes, 1 frontal lobe, and 1 parietal lobe) had strong and diffuse staining (3+) with four antibodies, which were consistent with those from automated IHC (clone D5F3, Ventana). (Fig. 3D).

4. Discussion

The molecular detection for NSCLC is welcome in a disease that has targeted therapeutic options. An evidence-based guideline, “Molecular Testing Guideline for the Selection of Lung Cancer Patients for ALK Tyrosine Kinase Inhibitors,” stated that all NSCLCs need to be evaluated for their ALK status [7]. In China, expert consensus on ALK-rearranged NSCLC diagnosis also indicated ALK screening should be performed on patients with potential ALK rearrangements [16]. The overall incidence of ALK gene rearrangement in NSCLC was estimated to be 3–13% in East Asian patients [17]. Therefore, it is reasonable to develop a more economical and valid ALK screening assay for as many NSCLC patients as possible. Although ALK FISH is the current reference standard for selecting patients for ALK inhibitor treatment [18], IHC is a population-wide screening in the routine work of every diagnostic pathology department. According to the molecular testing guideline [7], ALK IHC, if carefully validated, is accepted as a pre-screening method before FISH testing. There have been a number of reports about conventional manual IHC with ALK antibodies on NSCLC. However, since ALK protein was expressed at lower levels, the antibodies often did not show excellent sensitivity and specificity [2,8,14,15,19].

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Fig. 2. ALK expression on the excision specimens using the conventional manual IHC without amplification system. (A) Heterogeneous staining according to score 3+ in right tumor cells, but only score 1+ in left tumor cells (original magnification: 200×); (B) score 3+ with clone 1A4/1H7 (original magnification: 200×); (C) score 2+ with clone D5F3 (CST) (original magnification: 200×); (D) score 1+ with clone 5A4 (original magnification: 200×).

Fig. 3. (A) On the biopsy specimens, strong staining by automated IHC (clone D5F3, Ventana) (original magnification: 200×); (B) score 2+ by manual IHC with clone 1A4/1H7 (original magnification: 200×); (C) the weak cytoplasmic expression (score 1+) by manual IHC on the biopsy specimens (original magnification: 200×). (D) Diffuse cytoplasmic, homogeneous staining (score 3+) in the front lobe metastatic lung adenocarcinoma by manual IHC with 1A4, D5F3 (Ventana), D5F3 (CST), and 5A4 (original magnification: 200×).

Therefore, the optimal clinical strategy remains to be determined. In this study, we analyzed a cohort of 60 NSCLCs retrospectively by comparing the staining of four different ALK antibodies with conventional manual IHC and automated IHC (clone D5F3, Ventana). The results were confirmed by FISH analysis to evaluate the potential role of manual IHC as a detection method for ALK. The current results suggest that the application of conventional man-
In our study of 60 NSCLCs, we observed good consistancy between the protein status of ALK detected by automated IHC (clone D5F3, Ventana) and the results from FISH, as previously described [2,8,20]. As the OptiView amplification kit builds a molecular tree with DAB chromogen bound to an amplification multimer that is linked to multiple happens on top of the primary antibody, the signal intensity and sensitivity are stronger than with conventional staining, and false-positive cases does not increase.

### Table 3

<table>
<thead>
<tr>
<th>Number of NSCLC; ALK-rearranged NSCLC</th>
<th>Antibody clone; Working dilution</th>
<th>Detection system and signal amplification</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive predictive value</th>
<th>Negative predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gruberet al.</td>
<td>218; 20</td>
<td>1A4 (OriGene Techn.), 1:50</td>
<td>20/20, 100%</td>
<td>196/198</td>
<td>20/22, 90.0%</td>
<td>196/196, 100%</td>
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<td>Ying et al.</td>
<td>196; 63</td>
<td>D5F3 (CST), 1:50</td>
<td>63/63, 100%</td>
<td>126/133</td>
<td>63/70, 90%</td>
<td>126/126, 100%</td>
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<td>Selinger et al.</td>
<td>594; 7</td>
<td>D5F3 (CST), 1:100</td>
<td>7/7, 100%</td>
<td>581/587</td>
<td>7/13, 53.8%</td>
<td>581/581, 100%</td>
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<td>Zwaenepoel et al.</td>
<td>53; 30</td>
<td>D5F3 (CST), 1:50</td>
<td>29/30, 96.6%</td>
<td>20/23, 87%</td>
<td>29/32, 90.6%</td>
<td>20/24, 83.3%</td>
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<td>Fu et al.</td>
<td>173; 18</td>
<td>D5F3 (CST), 1:200</td>
<td>15/18, 83.3%</td>
<td>173/173</td>
<td>15/15, 100</td>
<td>155/158, 98.1%</td>
</tr>
<tr>
<td>Li et al.</td>
<td>161; 44</td>
<td>D5F3 (CST), 1:50</td>
<td>39/41, 95.1%</td>
<td>117/117</td>
<td>39/44, 88.6%</td>
<td>117/120, 97.5%</td>
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<td>Zhou et al.</td>
<td>366; 24</td>
<td>D5F3 (CST), 1:100</td>
<td>28/28, 100%</td>
<td>338/340</td>
<td>28/30, 93.3%</td>
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<td>303; 14</td>
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<td>Conde et al.</td>
<td>103; 47</td>
<td>5A4 (Novocastra), 1:20</td>
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<td>56/56, 100%</td>
<td>46/46, 100%</td>
<td>56/57, 98.2%</td>
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<td>Selinger et al.</td>
<td>594; 7</td>
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<td>7/18, 38.9%</td>
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<td>Savic et al.</td>
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<td>269/272, 98.0%</td>
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<td>Houg et al.</td>
<td>307; 12</td>
<td>5A4 (Novocastra), 1:10</td>
<td>12/12, 100%</td>
<td>293/295</td>
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<td>Zwaenepoel et al.</td>
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<td>22/23, 95.7%</td>
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<td>Blackhall et al.</td>
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<td>1201/1253</td>
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<td>Hutarew et al.</td>
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<td>280/289</td>
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<td>Hutarew et al.</td>
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<td>12/15, 85%</td>
<td>286/289</td>
<td>12/15, 80%</td>
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<td>McLeer-Fiorini et al.</td>
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<td>21/21, 100%</td>
<td>59/60, 98.3%</td>
<td>21/22, 95.5%</td>
<td>59/59, 100%</td>
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<td>Cabllic et al.</td>
<td>1832; 46</td>
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<td>Le Quesne et al.</td>
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<td>15/19, 78.9%</td>
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<td>Le Quesne et al.</td>
<td>29; 14</td>
<td>ALK1 (Dako), 1:20</td>
<td>11/14, 76.8%</td>
<td>11/14, 76.8%</td>
<td>11/14, 76.8%</td>
<td>15/18, 83.3%</td>
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<tr>
<td>Li et al.</td>
<td>101; 10</td>
<td>ALK1 (Dako), 1:100</td>
<td>10/10, 100%</td>
<td>69/91, 75.8%</td>
<td>10/32, 31.2%</td>
<td>69/69, 100%</td>
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<tr>
<td>Selinger et al.</td>
<td>594; 7</td>
<td>ALK1 (Dako), 1:10</td>
<td>7/7, 100%</td>
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<td>Li et al.</td>
<td>161; 44</td>
<td>ALK1 (Dako), 1:10</td>
<td>28/34, 63.6%</td>
<td>117/117</td>
<td>28/32, 87.5%</td>
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<td>7/14, 50%</td>
<td>286/289</td>
<td>7/10, 70%</td>
<td>286/293, 97.6%</td>
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IHC = immunohistochemistry; NSCLC = non-small cell lung carcinoma.
CST = cell signaling technology.

IHC and novel antibody (clone 1A4/1H7) may provide a reliable screening for identifying ALK-positive in NSCLCs.
[14]. These all guarantee the standardization of detection and the reliability of diagnosis. However, expensive experimental apparatus, such as automated immunohistochemical stainings for clinical screening are only available in just a few major pathology departments. This could prevent them from being used widely in local small- to medium-sized hospitals in China. Zhou et al. [8] described that all 26 manual IHC (3+) cases had been confirmed to actually harbor ALK rearrangements by FISH or PCR, and this resulted in 100% sensitivity and 100% specificity. In our study, ALK IHC analysis of the cases stained by four antibodies showed high concordance and 100% positive expected values compared with the results from the autostainer. We also observed that the 32 cases identified as ALK-rearranged demonstrated IHC 3+, IHC 2+, IHC 1+ or IHC−, which reflected and emphasized the heterogeneity of staining intensity and distribution with the modified semiquantitative graded criteria [8,21]. We suggest that ALK protein expression based on manual IHC, regardless of the percent and intensity of stained cells (including 1+, 2+, and 3+), would be potential ALK-positive NSCLCs. That is consistent with the research from the literatures [2,14]. Here, we present a diagnostic algorithm that manual IHC could be applied as an initial screening for the ALK protein status, and all staining positive cases including IHC 3+, 2+, and 1+ will need to be further verified by FISH, RT-PCR, or sequencing assay. On the other hand, several cases confirmed as ALK-rearranged NSCLC were assessed as negative by manual IHC in our cohort. Therefore, advanced molecular testing should be performed subsequently in IHC-negative cases with patients and considered to be at high risk for ALK-rearranged NSCLCs (such as young, non/light smokers, wild-type EGFR/KRAS, or solid lung adenocarcinoma).

On reviewing ALK antibodies, several different antibodies are commercially available for ALK IHC. Among them, clones 5A4 (Abcam/Novocastra), D5F3 (CST), and ALK1 (Dako) were most commonly studied [8,15,18,22–28]. Comparing primary antibodies is very difficult since there are very few published studies with comparative data where the different detection methodology and some variation in protein concentrations in various primary antibodies have resulted in inconsistent and unreliable staining. Further details are shown in Table 3. Li et al. [23] and Zhou et al. [8] had recommended manual IHC with clone D5F3 (CST) as the preferred combination for screening ALK-rearranged NSCLC. Hatare et al. [14] detected 14 cases harbored ALK-rearranged NSCLC with clone D5F3 (CST) by manual IHC, and showed a score of 3+ of 21%, a score of 2+ of 50% and a score of 1+ of 29% with 11 false-positive cases. Using the same antibody and staining procedure, Conkin et al. [29] revealed 3 ALK-positive cases with a score of 3+, a score of 2+ and a score of 1+ individually. Cabillic et al. [30] analyzed 1843 NSCLCs by automated IHC and clone 5A4 (Abcam), and the sensitivity was 72%. In the previous studies, poor sensitivity was obtained with clone 5A4 from Abcam, though excellent sensitivity was detected by Novocastra 5A4 [31–33]. Similar to our experiment with the same detection methodology, the staining sensitivity and intensity of four antibodies were evidently different from 56.3% to 93.8% and from 21.9% to 68.8%. The unsatisfactory data of the multicenter studies also highlighted the challenge of methodological variability and the importance of high affinity ALK antibody. We observed that 1A4/1H7 was a better option with higher staining intensity than the other three antibodies, detecting 30/32 ALK FISH-positive NSCLCs corresponding to a sensitivity and specificity of 93.8% and 100%, respectively. The latest literature from Gruber et al. [34] reported that 1A4 antibody had 100% sensitivity and 99.1% specificity, using a conventional IHC staining, with 17 cases revealing moderate to strong protein expression and 3 weak staining intensity cases, where it performed equally well as the Ventana DSF3 assay. These data reflected that clone 1A4/1H7 might be one of the preferred antibodies with predictive value in screening ALK-rearranged NSCLCs based on conventional manual IHC procedure without signal enhancement.

Most patients diagnosed with lung cancer were in advanced stages, and were either inoperable or having residual or recurrent disease after surgery. As in our practice, ALK status testing is required increasingly to be done on small biopsy samples. Abe et al. [20] and Houang et al. [18] concluded that patients who were ALK IHC-positive on biopsy specimens subsequently underwent surgical excision of the primary pulmonary or metastatic mass. When ALK IHC was performed on more than one sample from the same patient, it showed high concordant results. However, in the present study, ALK IHC in excision specimens had higher sensitivity and intensity when compared to biopsy specimens. One of the potential disadvantages of ALK IHC is that staining can be affected by pre-analysis factors such as differences in fixation or processing [14,18,35]. In the “guideline” [36], it was recommended that a fixation time of 6–12 h for small biopsy samples and 8–18 h for larger excision specimens with 10% formalin generally would give the best results. The in-house cases, both excision specimens and smaller biopsies, were fixed and performed with the same procedure. Another interpretive challenge is intratumoral heterogeneity. This study presented 25% specimens exhibited (from moderate to strong) heterogeneous staining using the automated IHC (clone D5F3, Ventana), and 40.6% heterogeneity (weak–moderate–strong intensity) using manual ALK IHC. In addition, we encountered four metastasized cases which had strongly diffuse staining in all four antibodies. Two of the patients had benefited from crizotinib therapy. Unfortunately, we were unable to compare ALK protein levels between the primary tumor and the metastatic lesion without the primary lung cancer specimens. Ali et al. [37] described a patient who suffered from the carinal lymph node metastasis after surgical resection of lung adenocarcinoma, and who presented ALK FISH translocation and ALK IHC strong expression both on the primary tumor and lymph node metastasis. These indicated that molecular detection of ALK expression in metastatic NSCLC had similar results compared with that in the primary cancer.

In conclusion, since lung cancer has become the most common malignant tumor and the leading cancer death in China, it is essential to develop a compatible testing at a reduced cost. We consider the conventional manual IHC with antibody clone 1A4/1H7 as the initial screening followed by ALK FISH confirmation to be a reliable, economical and population-wide application. This assay will enhance the quality and ensure the precision of ALK IHC test in the future.

Conflict of interest statement

The authors declare no conflict of interest.

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