Antibody 1A4 with routine immunohistochemistry demonstrates high sensitivity for ALK rearrangement screening of Chinese lung adenocarcinoma patients: A single-center large-scale study

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ABSTRACT

Objectives: The rearrangement of echinoderm microtubule-associated protein-like 4-analplastic lymphoma kinase (EML4-ALK) in non-small cell lung cancer (NSCLC) cells might be a promising therapeutic target. However, the low positive rate seeks a reliable and cost-effective method for ALK rearrangement prescreening. This study aimed to evaluate the application of a novel primary antibody 1A4 for routine ALK immunohistochemistry (IHC) test.

Materials and methods: Primary antibody 1A4 and DSF3 were used for the screening of 595 formalin-fixed, paraffin-embedded tissues of consecutive patients with lung adenocarcinoma for ALK-positive candidates. Ventana detection system and fluorescence in-situ hybridization (FISH) were used as reference methods.

Results: Among 595 cases, the protein expression statuses of 1A4 were 3+ (18), 2+ (50), 1+ (153), and 0+ (374), and those of DSF3 were 3+ (17), 2+ (18), 1+ (20), and 0+ (540). Ventana detection system and FISH test results were successfully obtained from 482 cases. A total of 298 specimens with 1A4 (+) showed 100% concordance with standard FISH results. All 58 FISH (+) cases were identified by antibody 1A4. Meanwhile, 14 and 5 were missed by antibody DSF3 with routine IHC and Ventana system, respectively. 1A4 with routine IHC had better sensitivity (100%, 75.9%, and 91.4%, respectively), but lower specificity (70.3%, 99.8%, and 100%, respectively), than DSF3 with routine IHC and Ventana system.

Conclusion: The novel antibody 1A4 used as a prescreening method may help to reduce the false-negative rearranged ALK status if FISH or reverse transcription polymerase chain reaction results were used for validation.

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1. Introduction

Non-small cell lung cancer (NSCLC) is the most common cause of cancer-related deaths in both men and women in China. The fusion gene EML4-ALK, the echinoderm microtubule-associated protein-like 4 (EML4) gene and the anaplastic lymphoma kinase (ALK) gene, was discovered in a subset of patients with lung adenocarcinoma [1]. Recent studies have suggested that the rearrangement of EML4-ALK in NSCLC cells might be a promising therapeutic target [2].

In a Phase I study, crizotinib (Xalkori; Pfizer, NY, USA), a dual ALK and Met inhibitor, showed a great improvement in overall response rate (ORR) and progression-free survival (PFS) in patients carrying the ALK translocation [3,4]. Phase III trials showed that, when compared with chemotherapy, crizotinib used in both first- and second-line treatments significantly prolonged PFS, increased response rates, and improved the quality of life in patients with advanced and ALK-positive NSCLC [5,6]. However, merely 3–7% of adenocarcinoma patients with ALK-rearranged tumors could benefit from crizotinib treatment [1,7]. Therefore, a reliable and cost-effective method for ALK rearrangement screening will be of great clinical significance.

Fluorescent in-situ hybridization (FISH), immunohistochemistry (IHC) with antibody DSF3 and Ventana system, and reverse transcription polymerase chain reaction (RT-PCR) are three methods that have always been recommended for ALK rearrangement detection. As the gold standard for ALK rearrangement detection,
FISH is well known as the first method (the only one before June 2015) using the Vysis ALK Dual Color Break Apart FISH Probe Kit (Abbott Molecular, IL, USA), which had been approved by the US Food and Drug Administration (FDA) in 2011 along with crizotinib. However, the signal instability, scoring difficulties, and expensive FISH probes have impeded its application in nonspecialized laboratories [6]. The poor qualities of RNA obtained in formalin-fixed paraffin-embedded (FFPE) tissues and limitation on unknown rearrangement types have posed a great challenge for RT-PCR-based ALK fusion-gene detection [9]. IHC is relatively inexpensive, convenient, and perfectly adapted for daily ALK rearrangement screening. Depending on the specific antibody, the sensitive detection system, and the experience of the pathologists, the D5F3/Ventana system has achieved a great improvement in diagnostics of ALK-rearranged NSCLC patients [10,11]. However, the Ventana Benchmark XT System, with the primary antibody D5F3, Optiview DAB Detection Kit, and Optiview Amplification Kit, is so expensive that it is affordable only for a minority of pathology departments in the world. Thus, prescreening of ALK-rearranged candidates by the IHC test with cost-effective and sensitive antibodies would be a good choice.

This study aimed to evaluate the application of a novel primary antibody 1A4 for routine ALK immunohistochemistry test. D5F3, detected by both traditional IHC and Ventana system, was used in this study for a direct comparison of the sensitivity and specificity with 1A4. ALK FISH status was used as the reference group.

2. Materials and methods

2.1. Patients

This study was approved by the Clinical Ethics Committee of Daping Hospital and Research Institute of Surgery, Third Military Medical University. A total of 595 FFPE tissues of consecutive patients with lung adenocarcinoma were enrolled in this study. They were randomly selected after the diagnosis of lung adenocarcinoma, and none of them had received ALK inhibitor treatment or chemotherapy before surgery or biopsy. The clinical data and smoking history of the patients were obtained from their medical records.

2.2. Immunohistochemistry

A novel antibody 1A4 (Origene, MD, USA) and D5F3 (Ventana Medical Systems, Inc., AZ, USA) were tested by the conventional IHC. The incubation of primary antibodies was performed on 4-μm-thick, formalin-fixed, paraffin-embedded (FFPE) tissues: the slides were deparaffinized and pretreated with 1 mmol/L ethylenediaminetetraacetic acid (EDTA) at pH 9.0 in a high-pressure cooker for 3 min and then treated with 3% hydrogen peroxide for 10 min. The primary antibodies 1A4 and D5F3 were applied to the slides and then incubated in the hydrated chamber at 4°C overnight. Next day, the slides were washed, stained using the conventional 3,3′-diaminobenzidine staining, and reviewed under a microscope by two pathologists independently. The positive and negative control sections (validated by FISH and RT-PCR) were used and reviewed at the same time. The slides were scored as strong staining (3+), moderate staining (2+), faint staining (1+) and no staining (0).

A fully automated Ventana Benchmark XT System (Ventana Medical Systems, Inc., AZ, USA), with the D5F3 antibody, the Optiview DAB Detection Kit, and the Optiview Amplification Kit, was used according to the manufacturer’s recommendations [10]. D5F3/Ventana positive staining was classified as tumors with any strong, granular, cytoplasmic staining; while tumors with faint cytoplasmic staining were regarded as negative.

2.3. Fluorescence in-situ hybridization

FISH was performed on unstained 4-μm-thick FFPE specimens using the Vysis ALK Dual Color Break Apart FISH Probe (Abbott Molecular, IL, USA), according to the manufacturer’s instructions. The slides were read on a fluorescence microscope (BX51; Olympus, Japan) and evaluated by two experienced pathologists independently. Tumor cells containing at least one pair of green and orange signals, split apart by ≥2 signal diameters, or a single orange signal without the corresponding green signal, were diagnosed as positive for ALK gene rearrangement. The criteria of 15% break-apart signals or orange single signals isolated in 10 tumor cells were used as the ALK-positive cut-off levels [12].

2.4. Statistical analysis

The relationship of ALK gene rearrangement rates with clinical characteristics was analyzed using the Pearson’s χ² test. P < 0.05 was considered statistically significant. All analyses were performed using the SPSS software (version 18.0; SPSS Inc., IL, USA).

3. Results

3.1. Clinical features of tumors

The clinical characteristics of the 595 patients with lung adenocarcinoma are shown in Table 1. The population consisted of 350 males (58.9%) and 245 females (41.1%), with a mean age of 60.1 years (range 24–87 years). Based on their smoking history, 361 patients were further categorized as never-smokers (60.7%) and 234 as smokers (39.3%). A total of 595 tumor samples consisted

Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of Patients (%)</th>
<th>ALK (FISH) Positive (%)</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>350 (58.9)</td>
<td>35/276 (12.7)</td>
<td>0.256</td>
<td>0.613</td>
</tr>
<tr>
<td>Females</td>
<td>245 (41.1)</td>
<td>23/206 (11.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>361 (60.7)</td>
<td>35/283 (12.4)</td>
<td>0.072</td>
<td>0.788</td>
</tr>
<tr>
<td>Smokers</td>
<td>234 (39.3)</td>
<td>23/199 (11.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specimen type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgical specimens</td>
<td>235 (39.5)</td>
<td>32/234 (13.7)</td>
<td>0.905</td>
<td>0.341</td>
</tr>
<tr>
<td>Biopsies</td>
<td>276 (46.4)</td>
<td>18/171 (10.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pleural effusion cell blocks</td>
<td>21 (3.5)</td>
<td>3/21 (14.3)</td>
<td>0.071</td>
<td>0.79</td>
</tr>
<tr>
<td>Metastases†</td>
<td>63 (10.6)</td>
<td>5/56 (8.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>595</td>
<td>58/482 (12.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FISH: Fluorescence in-situ hybridization.

“%” in the second column refers to all 595 patients.

† Metastases in brain, bone, pleura, and cervical lymph node.
of 235 surgical specimens (39.5%), 276 biopsies (46.4%), 21 pleural effusion cell blocks (3.5%), and 63 metastases (brain, bone, pleura, and cervical lymph node) (10.6%).

3.2. ALK rearrangement detection by FISH

Among the 595 patients, FISH results were successfully obtained only from 482 patients, and failed for the remaining 113 specimens. A total of 58 patients (58/482, 12.0%) harboring rearranged ALK genes were identified by the Vysis ALK Dual Color Break Apart FISH Probe (Table 1). The ALK-positive rate in males and females, and smokers and non-smokers were 12.7% (35/276) and 11.2% (23/206), and 11.6% (23/199) and 12.4% (35/283), respectively, with no significant differences. The ALK-positive rate in surgical specimens and biopsies was 13.7% (32/234) and 10.5% (18/171), respectively, with no significant differences either. Pleural effusion was embedded as cell blocks for subsequent FISH and IHC analysis. The ALK-positive rate of cell blocks was 14.3% (3/21) higher (but not significantly) than other metastases (8.9%, 5/56), comprising brain, bone, pleura, and cervical lymph node.

3.3. The primary antibody 1A4 showed a higher sensitivity than D5F3

A total of 595 patients had successful routine IHC staining for 1A4 and D5F3 protein expression (Table 2). The protein expression statuses of 1A4 were 3+ (18), 2+ (50), 1+ (153), and 0+ (374), and those of D5F3 were 3+ (17), 2+ (18), 1+ (20), and 0+ (540). For comparison, Ventana detection system (D5F3) and FISH test results were successfully obtained from 482 cases (Table 3). A total of 298 specimens with 1A4 (−) showed 100% concordance with standard FISH results. All 58 FISH (+) cases were identified by antibody 1A4. Meanwhile, 14 and 5 were missed by antibody D5F3 with routine IHC and Ventana system, respectively (Fig. 1). The sensitivity of 1A4 with routine IHC, D5F3 with routine IHC, and Ventana system was 100%, 75.9%, and 91.4%, respectively.

3.4. D5F3 showed a higher specificity than 1A4

Of 424 FISH (−) cases, 298 were verified by 1A4 (−) with routine IHC; 423 and 242 were validated by D5F3 with routine IHC and Ventana system, respectively (Table 3). The specificity of ALK rearrangement detection by FISH.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>ALK protein status of 595 patients detected by 1A4 and D5F3 with routine IHC.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1A4</td>
</tr>
<tr>
<td>0+</td>
<td>374</td>
</tr>
<tr>
<td>1+</td>
<td>138</td>
</tr>
<tr>
<td>2+</td>
<td>27</td>
</tr>
<tr>
<td>3+</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>540</td>
</tr>
</tbody>
</table>

Fig. 1. ALK rearrangements detected by 1A4 with routine IHC, D5F3 with routine IHC, and D5F3 with Ventana system and FISH assay.
Case A: 1A4 (−), D5F3 (−), D5F3 Ventana (−), and FISH (−); Case B: 1A4 (+), D5F3 (−), D5F3 Ventana (−), and FISH (+); Case C: 1A4 (+), D5F3 (−), D5F3 Ventana (+), and FISH (+); Case D: 1A4 (+), D5F3 (+), D5F3 Ventana (+), and FISH (+).
Table 3

<table>
<thead>
<tr>
<th>ALK status by FISH</th>
<th>N</th>
<th>1A4 with routine IHC</th>
<th>DSF3 with routine IHC</th>
<th>DSF3 with Ventana</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3+</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>+</td>
<td>58</td>
<td>17</td>
<td>23</td>
<td>18</td>
</tr>
<tr>
<td>–</td>
<td>424</td>
<td>0</td>
<td>19</td>
<td>107</td>
</tr>
<tr>
<td>Total</td>
<td>482</td>
<td>17</td>
<td>42</td>
<td>125</td>
</tr>
</tbody>
</table>


rangement test by DSF3 with routine IHC or Ventana system was higher than that of 1A4 with routine IHC, 99.8% (423/424) or 100% (424/424) vs 70.3% (298/424), respectively.

4. Discussion

The discovery of ALK rearrangements in the subset of NSCLC [1] has rapidly led to the validation of the ALK inhibitor crizotinib in a phase III trial in which patients were enrolled on the basis of the positive FISH results [3,13]. The FISH assay was soon approved by the Food and Drug Administration (FDA) in the United States as the first method to detect ALK rearrangement in NSCLC. The rate of FISH ALK-positive cases was 3–7% and 10% with previous data reported in white and Chinese people, respectively [14–17]. In this study, 12.0% patients with lung adenocarcinoma patients (58/482) were identified as having rearranged ALK genes by the Vysis ALK Dual Color Break Apart FISH Probe. A higher ALK-positive rate of Chinese than white people may suggest an ascertainment bias or a geographical variation. ALK-rearranged patients in this study showed no significant correlation with smoking history, sex, specimen type, and metastases.

FISH and RT-PCR are currently two specific and generally acceptable methods for ALK rearrangement detection. However, considering the low frequency of ALK rearrangements and the high incidence of NSCLC, it is not feasible to use these two methods as a routine test for ALK-positive tumors, due to the cost, the required expertise, and the labor-intensive manipulation of the test [18]. Immunohistochemistry is a valuable and cost-effective screening method for potential ALK-positive patients, which could reduce the number of cases requiring FISH or RT-PCR tests [19]. Different ALK antibodies (ALK1, 5A4, and DSF3) for IHC assay have been evaluated, and the results are controversial [20]. The data from other reports [10,21,22] showed that DSF3 or 5A4 antibody, but not ALK1, was more sensitive and specific to genetic testing in the diagnosis of ALK-rearranged lung adenocarcinoma. However, the sensitivity of DSF3 or 5A4 was still not as satisfactory as FISH assay, even though the OptiView DAB Detection and OptiView Amplification Kit were used together [23,24].

Gruber et al. [25] showed that the novel antibody 1A4 detected 100% ALK-positive patients with lung adenocarcinoma in Germany, which is higher than 95% in the case of DSF3/Ventana system. This is the only study focused on applying 1A4 antibody to ALK rearrangement screening. Nevertheless, evidence of the use of 1A4 for detecting ALK in Chinese patients with lung adenocarcinoma is still lacking. Antibody 1A4 was used in this study for ALK rearrangement screening compared with the frequently recognized antibody DSF3 (analyzed by routine IHC and Ventana system). A total of 595 lung adenocarcinoma cases were enrolled in this study. The results showed that 1A4 antibody yielded higher sensitivity (100%) on ALK rearrangement detection compared with DSF3 antibody, no matter with routine IHC (75.9%) or Ventana system (91.4%). This was consistent with the recent report by Rodig et al. [26]: approximately 20% of FISH–positive cases remained IHC (ALK1) negative. The use of the DSF3 antibody in 17 FISH–positive/IHC-negative samples of the Bordeaux series in a second IHC testing allowed the detection of ALK expression in only five cases, whereas 12 cases remained negative. This indicated that the sensitive antibody 1A4 may help to reduce the false-negative rearranged ALK tests if used as the prescreening method and followed by FISH or RT-PCR validation.

When 1A4 is used as a prescreening antibody, cases showing any ALK expression by IHC (3+, 2+, and 1+) should be tested by FISH or RT-PCR for the validation of ALK positivity. Other cases with no 1A4 expression would be considered as ALK negative.

Conflict of interest

We report no conflicts of interest and ethical adherence in this work. And we declare that neither the entire paper nor any part of its content has been published or been accepted elsewhere, and it is not being submitted to any other journal either.

Acknowledgement

We would like to thank He Xiao for the assistance on statistical analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.jlungcan.2016.02.014.

References


