Diagnostic value of a novel fully automated immunohistochemistry assay for detection of ALK rearrangement in primary lung adenocarcinoma

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Background: To evaluate the diagnostic value of a novel fully automated immunohistochemistry (IHC) assay for detection of anaplastic lymphoma kinase (ALK) fusion in a large number of ALK-positive lung adenocarcinoma (ADC) patients.

Patients and methods: We tested 196 lung ADCs for ALK rearrangement by two IHC assays (Ventana pre-diluted ALK D5F3 antibody with the Optiview DAB IHC detection kit and Optiview Amplification kit, D5F3 by Cell Signaling Technology (CST) with Ultraview DAB detection kit by Ventana), fluorescence in situ hybridization (FISH) and real-time reverse transcription–PCR (RT–PCR). CST ALK IHC was scored using the scoring scheme of 0, no staining; 1+, faint; 2+, moderate; and 3+, strong cytoplasmic reactivity in ≥10% of tumor cells. As for Ventana IHC, a binary scoring system (positive or negative for ALK status) was adopted for evaluating the staining results.

Results: Among 196 cases tested, 63 (32%), 65 (33%), 70 (36%), and 69 (35%) cases were ALK positive by FISH, Ventana IHC, CST IHC, and RT–PCR, respectively. The sensitivity and specificity of Ventana IHC were 100% and 98%, respectively. Two Ventana IHC-positive cases, which were also CST IHC score of 3+, showed FISH negative, but their ALK rearrangement was confirmed by RT–PCR and direct sequencing. The sensitivity and specificity of CST IHC with staining intensity score of 1 + or more were 100% and 95%, respectively. Five (25%, of 20) patients with CST IHC score of 1+ were both FISH and RT–PCR negative. The sensitivity and specificity of RT–PCR for detection of ALK fusion were 98% and 95%, respectively. The total accordance rate between ALK RT–PCR and Ventana IHC was 97%.

Conclusions: The novel fully automated IHC assay is a reliable screening tool in routine pathologic laboratories for identification of patients with ALK rearrangement for targeted therapy in lung ADC.

Key words: ALK, immunohistochemistry, fluorescence in situ hybridization, lung cancer, targeted therapy

Introduction

Lung cancer is the most frequent cause of cancer-related death worldwide, accounting for about 1.4 million deaths per year [1]. Although conventional chemotherapy remains the mainstay of treatment of the majority of patients with advanced non-small-cell lung cancer (NSCLC), the identification of multiple genetic abnormalities which drive oncogenic signaling pathways within cancer cells has led to the development of new targeted therapies in a subset of patients with NSCLC [2, 3]. For example drugs that target epidermal growth factor receptor (EGFR) have been proven more effective than cytotoxic chemotherapy in advanced NSCLC patients with sensitizing EGFR mutations [2, 3]. More recently, a similarly marked response to an anaplastic lymphoma kinase (ALK) inhibitor was demonstrated in patients with ALK fusion-bearing NSCLC [4, 5].

The echinoderm microtubule-associated protein-like 4 – ALK (EML4-ALK) fusion in NSCLC was first described in 2007 [5]. This fusion results from a small inversion within chromosome 2p, resulting in the expression of a chimeric tyrosine kinase in which the N-terminal half of the EML4 is fused to the intracellular ALK kinase domain. Although EML4 is the predominant fusion partner, other fusion partners have also been reported in lung cancer, including TFG [6], KIF5B [7], and KLC1 [8]. The incidence of ALK gene rearrangement appears to range from 2% to 7%, and they are rarely coincident with EGFR, KRAS mutations [5, 9, 10]. Recently, results from clinical trials evaluating an ALK inhibitor, Crizotinib, in patients with ALK positive locally advanced or metastatic NSCLC demonstrated promising results [4]. However, lung cancer with the ALK fusion constitutes only a small fraction of lung cancers, therefore, efficient determination of ALK status in NSCLC patients is critical for directing patient care.
Currently, fluorescent in situ hybridization (FISH), immunohistochemistry (IHC), and real-time reverse transcription–PCR (RT–PCR) are commonly used to detect the ALK fusion. Each of these methods has its own advantages and disadvantages. Although FISH is currently the gold standard method used in clinical trials to detect fusion gene, and it was the first FDA-approved method for detecting the ALK fusion, IHC analysis is technically easy because it is integrated into routine pathological diagnosis. Recent studies indicate that IHC is sensitive and specific for determination of ALK status, and is a viable alternative to ALK FISH [11–16]. However, studies comparing IHC and with FISH have used different clones with different detection systems and scoring methods, and only small number of ALK-positive cases were collected in these studies, due to its low incidence. In this study, we introduced a fully automated IHC assay to determine ALK status, together with another reported IHC assay, FISH, and RT–PCR in a large collection of ALK-positive cases, and compared the specificity and sensitivity of this novel IHC assay with other methods for the detection of ALK fusion in patients with primary lung ADC.

**patients and methods**

**patients**

All included patients had received curative surgery at the Cancer Hospital, Chinese Academy of Medical Sciences (CAMS), Beijing, China, between August 2011 and July 2012. All these tumor samples were fixed in 10% neutral buffered formalin for 24–48 h and embedded in paraffin and routinely diagnosed as primary lung ADC. All these samples were previously screened for ALK fusion by FISH, including 63 ALK-positive and 133 ALK-negative cases. The study protocol was approved by the Institute Review Board of the Cancer Hospital, CAMS. Tissue microarray blocks were built to perform IHC and FISH assays (for more details, see the supplementary Appendix, available at *Annals of Oncology* online).

**fluorescence in situ hybridization**

FISH analysis was carried out with the Vysis LSI ALK Dual color, Break Apart Rearrangement Pobe (Vysis/Abbott, Abbott Park, IL) according to the manufacturer’s instructions. Four cases that failed to get satisfactory signals on TMA were tested further on whole sections of the corresponding tumor blocks by FISH.

**immunohistochemistry**

Two automated IHC assays were carried out in this study.

One assay (CST IHC) used Rabbit monoclonal ALK antibody (clone DSF3, Cell Signaling Technology) diluted 1 : 50 using a Ventana Ultraview DAB detection kit in a Ventana Benchmark XT stainer (Ventana Medical Systems, Tucson, AZ). ALK IHC was scored using the scoring scheme proposed as follows: 0, no staining; 1+, faint cytoplasmic reactivity without any background staining; 2+, moderate cytoplasmic reactivity; and 3+, granular cytoplasmic reactivity of strong intensity in ≥10% of tumor cells.

The other assay (Ventana IHC) was a fully automated IHC assay developed by Ventana recently, using the pre-diluted Ventana anti-ALK (DSF3) Rabbit monoclonal primary antibody, together with the Optiview DAB IHC detection kit and Optiview Amplification kit on the Benchmark XT stainer. Each case was also stained with a matched Rabbit Monoclonal Negative Control Ig antibody. According to the manufacturer’s scoring algorithm, binary scoring system (positive or negative for ALK status) was adopted for evaluating the staining results. Neoplastic cells labeled with the ALK IHC assay are evaluated for presence or absence of the DAB signal. Presence of strong granular cytoplasmic staining in tumor cells (any percentage of positive tumor cells) was deemed to be ALK positive, while absence of strong granular cytoplasmic staining in tumor cells was deemed to be ALK negative.

**real-time RT–PCR**

The EML4-ALK fusion mRNA was readily detected by PCR using AmoyDx EML4-ALK Fusion Gene Detection Kit (Amoy Diagnostics, Xiamen, China) according to manufacturer’s instruction (see the supplementary Appendix, available at *Annals of Oncology* online, for more details). The amplified PCR products from some samples were subjected to direct sequencing, using AB3500xl DNA Sequencer (Applied Biosystems).

**results**

**ALK fluorescence in situ hybridization**

One hundred and ninety-six lung ADCs were included in this study, consisting of 108 female and 88 male patients. The mean age is 57.4 years (range, 24–81 years). Of these, 63 (32%, of 196) demonstrated an ALK rearrangement (FISH positive), and 133 cases were FISH negative (Figure 1). The FISH-positive cases included 35 women and 28 men with mean age of 52.5 years (range, 24–76 years). On FISH examination, split pattern was observed in 45 cases (71%) and unbalanced rearrangement, characterized by a loss of the 5’ probe, was shown in 18 cases (29%). Twelve cases (6%, of 196) showed average green or orange signals of more than three per cell.

**ALK immunohistochemistry**

By CST IHC assay, ALK protein was expressed in 70 (36%) tumors in 196 patients, including 20 (10%), 35(18%), and 15(8%) cases of 1+, 2+, and 3+, respectively (Figure 1 and supplementary Table S1, available at *Annals of Oncology* online).

By Ventana IHC assay, ALK protein was expressed in 65 (33%) tumors in 196 patients. All patients with CST by Ultraview IHC score of 2+ or 3+ (n = 50) were Ventana IHC positive and all patients with score of 0 (n = 126) were Ventana IHC negative. Among patients with CST IHC score of 1+, 5 (of 20) were Ventana IHC negative.

**ALK real-time RT–PCR**

Using real-time RT–PCR, 69 cases (35%, of 196) were identified to have EML4-ALK rearrangement, including 52 cases of EML4-ALK fusion variants 1/2/3a/b, and 2 cases of variants 5a/5b/5/8. Fifteen cases were both positive for Variants 1/2/3a/b and variants 5a/5b/5/8.

**correlation of ALK FISH, IHC, and real-time RT–PCR**

As shown in supplementary Table S1, available at *Annals of Oncology* online, of 50 patients with CST by Ultraview IHC score of 2+ or 3+, 48 (96%) were FISH positive and all patients with score of 0 (n = 126) were FISH negative. Among patients with CST IHC score of 1+, 15 (75.0%, 15 of 20) were FISH positive. The sensitivity and specificity of ALK CST IHC with staining intensity score of 1+ or more were 100% and 95%, respectively. The sensitivity and specificity of ALK CST IHC
with staining intensity score of 2+ or more were 76% and 98%, respectively.

Of 65 patients with Ventana IHC ALK positive, 63 (97%) were FISH positive and all patients with IHC ALK negative were FISH negative. The sensitivity and specificity of ALK Ventana IHC were 100% and 98%, respectively.

Sixty-two (90%, of 69) patients detected as positive by RT–PCR were FISH positive, while 126 (99%, of 127) patients with ALK negative by RT–PCR were FISH negative. The sensitivity and specificity of RT–PCR for detection of ALK fusion were 98% and 95%, respectively.

Of 69 ALK RT–PCR-positive patients, 64 (93%) were Ventana IHC positive, while 126 (99%, of 127) ALK RT–PCR-negative cases were Ventana IHC negative (supplementary Table S2, available at Annals of Oncology online). The total accordance rate between ALK RT–PCR and Ventana IHC was 97%.

Figure 1. Detection of ALK fusion in lung cancer patients by immunohistochemistry (IHC), FISH, and real-time RT–PCR assays. (A–D) Semiquantitative IHC staining using an antibody to ALK (D5F3 by Cell signaling [Billerica, MA] with Ventana Untraview DAB detection kit). (A) score 0/negative showing no staining; (B) score 1+ showing faint cytoplasmic reactivity without any background staining; (C) score 2+ showing moderate smooth cytoplasmic staining; and (D) score 3+ showing intense granular cytoplasmic staining in ≥10% of tumor cells. Original magnification ×200. (E–H) Ventana IHC assay revealed strong expression of ALK in all ALK-fusion-positive patients and no expression in ALK-fusion-negative patient. Original magnification ×200. (I–L) FISH carried out with Vysis LSI ALK Dual color Break-Apart FISH probes detected ALK fusion as split red and green signals. (I) FISH-negative case showing intact two fused signals per nucleus. (J and K) FISH-positive cases representing split (red and green) signals. (L) FISH-positive case demonstrating isolated red signal. Original magnification ×1000. (M–P) Graphs from real-time RT–PCR showing change in the normalized reporter signal (delta Rn) against PCR cycle number for reaction 1 of AmoyDx EML4-ALK fusion gene detection kit.

The details of discrepant cases for ALK fusion detection among IHC, FISH, and RT–PCR were shown in supplementary Figure S1 and Table S3, available at Annals of Oncology online).

discussion

In this study, we introduced a novel fully automated IHC assay developed by Ventana and retrospectively compared it with another high affinity antibody, FISH, and RT–PCR for the detection of ALK rearrangement in a large cohort of ALK-positive cases, together with negative cases. Using FISH as the standard procedure, we demonstrated that the novel fully automated IHC assay using pre-diluted Ventana anti-ALK (D5F3) Rabbit monoclonal primary antibody, together with the Optiview DAB detection and Amplification kit, is a highly sensitive (100%) and specific (98%) method for detection of the ALK rearrangement in primary lung adenocarcinoma. For these
two IHC-positive but FISH-negative cases, their ALK rearrangements were confirmed by RT–PCR. The high sensitivity and specificity of the IHC assay enables a reproducible, easy binary scoring system (positive or negative for ALK status) for evaluating the staining results.

Identification of appropriate patient population with reliable screening methods is the key to the overall success of tumor targeted therapies. Various methods including FISH, IHC, and RT–PCR can be used for the detection of ALK rearrangements, and each of them, inevitably, has its own advantages and disadvantages. In this study, we compared all three methods to evaluate their specificity and sensitivity for detection of ALK fusion, including the reported high affinity antibody CST D5F3. A total of 196 patients with lung ADC were tested by all methods, including 63 ALK-positive cases screened previously by FISH. To the best of our knowledge, this is the largest number of ALK-positive cases involved in the published literature.

Although a gold standard method for ALK-positive NSCLC had not been established, ALK positivity by FISH has been used as an eligibility criterion in clinical trials with crizotinib [4]. The break-apart probe allows detection of rearrangements, independent of the fusion partners or specific breakpoint. However, ALK gene alteration in NSCLC is an intrachromosomal rearrangement; thus, the split signal of the break-apart probe is relatively close, leading to difficult interpretation of ALK FISH in NSCLC. It has been pointed out that FISH as an initial screening tool did not detect all cases with ALK-positive NSCLC, because it can be misinterpreted as normal [17]. Similar to the FISH assay, IHC can detect ALK independent of the fusion partner. Several studies have been showed that variable results of ALK protein expression were detected by IHC with ALK antibodies in lung cancer, depending on the tissue preparation, the antibody affinity, the sensitivity of detection system used, the scoring system, and experience of the scorer [11, 13–15, 18, 19]. Due to the low level of EML4–ALK transcriptional activity, early studies with commercial ALK antibodies and standard protocol showed that IHC was specific but not sensitive for the detection of lung cancer with ALK rearrangement [20]. In contrast, using the highly sensitive detection methods in combination with high affinity antibodies, which were both developed recently, IHC can effectively detect ALK fusion protein in lung ADCs with high sensitivity and specificity, especially with antibodies of 5A4 and D5F3 [11, 15, 16, 20]. Multiplex RT–PCR system has been the usual screening strategy applied for ALK gene rearrangements [21]. The presence of fusion transcripts as detected by RT–PCR provides direct evidence of chromosomal translocation. However, it requires high-quality RNA, which is difficult from FFPE samples. Furthermore, RT–PCR can only detect fusion transcripts with known fusion partners.

Given that IHC is a routine methodology in most pathology laboratories to detect a protein of interest, it is desirable to establish a sensitive and accurate detection method for ALK fusion protein based on IHC. In this study, we demonstrated that both CST and Ventana IHC assays showed 100% of sensitivity, when compared with FISH results. However, the Ventana IHC showed a higher specificity than that of CST IHC (98% versus 95%). There was a correlation between the semiquantitative IHC score (1+, 2+, 3+) of CST IHC and the ALK-positive cases by FISH. In 20 CST IHC 1+ cases, five cases showed false-positive, which were ALK-negative confirmed by both FISH and RT–PCR. All 50 CST IHC 2+/3+ cases were confirmed to harbor ALK rearrangement either by FISH or by RT–PCR. This discrepancy was due to the difficulty to differentiate true weak staining with background or nonspecific staining in score 1+ cases. For Ventana IHC assay, although using the same antibody clone D5F3, when combined with a ultrasensitive detection system, all positive cases showed diffuse and strong immunoreactivity, resulting in 100% of specificity, when ALK-positive was defined as FISH or RT–PCR positive, and no semiquantitative score (1–3+) is necessary. These results demonstrated that, besides high affinity antibody, a higher sensitivity detection system was very important for the detection of ALK rearrangement. The strong staining in ALK-positive cases would be extremely helpful in avoiding interobserver variation in the assessment of ALK IHC status. No staining was observed in normal lung tissue, except nonspecific staining in macrophage cells, which is consistent with the biological fact that ALK protein is not constitutively expressed in normal lung tissue. Twelve (6%) cases showed ALK copy number gains in our cases. Several works have been reported to evaluate ALK copy number by FISH, with various incidences [17, 22]. These differences may be explained in part by the materials (TMA, biopsies or whole tumor sections) used and the ethnic groups studied. The clinical significance of ALK copy number gain in lung need to be further studied.

It has been reported that some of the ALK IHC-positive tumors that had been confirmed by RT–PCR showed no break-apart FISH signals [17, 20]. In our study, two cases demonstrated false-negative FISH results, despite repeat testing. Those two cases showed strong staining by both two IHC assays and RT–PCR positive. Because the EML4 and ALK loci are mapped relatively close on chromosome 2p, the subtle changes in fluorescent signal, caused by intrachromosomal inversion in some positive cases, might be difficult to interpret and might lead to false-negative results. The limited probe separation in such cases reduces the sensitivity of FISH assay. One NSCLC patient with complex ALK rearrangement that was negative by FISH analysis has been reported to be crizotinib-sensitive [23]. Real-time RT–PCR is a fast and sensitive method for detection of expressed known EML4–ALK fusion variants for which specific primers have been designed [21]. In this study, the sensitivity and specificity of RT–PCR for detection of ALK fusion were 98% and 95%, respectively. Twelve cases showed mixed ALK fusion patterns. We found that 8 of the 196 cases screened had inconsistent RT–PCR and FISH results. One FISH-positive case, which was also IHC-positive, showed negative result by RT–PCR, suggesting a new ALK fusion partner might be involved in this case, and needs to be further analyzed. Five FISH-negative cases showed positive results by RT–PCR, and they were also IHC-negative. RT–PCR results were confirmed by direct sequencing of the PCR products. It is difficult to determine whether these cases are truly positive for the ALK fusion in the transcriptional level, but no translation happens, or whether it was due to tumor heterogeneity.

In summary, we report that the fully automated IHC is a sensitive and specific screening method to detect ALK fusion in lung cancer.
rearrangement in lung cancer. IHC would be served as an effective and rapid detection method in routine pathologic laboratories for the identification of suitable candidates for ALK-targeted therapy. Our study demonstrated that some ALK IHC-positive but FISH-negative lung cancers did harbor the translocation events as confirmed by RT–PCR. Thus, this subgroup of patients should also benefit from ALK inhibitory therapy. Further clinical trials are required to address the predictive value of ALK IHC in these patients.

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disclosure
The authors have declared no conflicts of interest.

references