Clinical Characteristics and Outcomes of Patients with Primary Lung Adenocarcinoma Harboring ALK Rearrangements Detected by FISH, IHC, and RT-PCR

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Abstract

EML4-ALK is a new driver gene of non-small cell lung cancer and a target of crizotinib. The objectives of this study were to determine the frequency of ALK rearrangements in a large cohort of patients with primary lung adenocarcinoma and to analyze the association of ALK rearrangements with clinicopathological characteristics and clinical outcomes. The roles of fluorescence in situ hybridization (FISH), Ventana immunohistochemistry (IHC), and reverse transcriptase polymerase chain reaction (RT-PCR) in the detection of ALK rearrangements were evaluated. The ALK rearrangement was detected in 430 specimens from individual patients with primary lung adenocarcinoma using FISH and Ventana IHC based on tissue microarrays. The EGFR status was detected in all of the specimens through DNA sequencing. An RT-PCR was performed on 200 of the specimens and confirmed by sequencing. Of the 430 patients, 46 (10.7%) harbored ALK rearrangements. The ALK rearrangements were associated with a younger age and the EGFR wild type in comparison with ALK-negative patients. The sensitivity and specificity of the Ventana IHC were 100% and 98.2%, respectively, and the concordance rate between the FISH and the Ventana IHC was 98.4%. The sensitivity and specificity of RT-PCR were 95.5% and 87.0%, respectively, and the concordance rate between the FISH and the RT-PCR was 89.0%. The Cox analysis indicated that an early stage and EGFR-activating mutations were independently associated with a longer OS. This study demonstrated that ALK rearrangements are associated with a younger age and the EGFR wild type rather than with other clinicopathological factors. Although the FISH and Ventana IHC have better concordance, and RT-PCR is a more sensitive method and can identify different variants or partners, the IHC and RT-PCR need to be further evaluated in clinical trials to identify their roles in guiding patients’ targeted therapy using crizotinib.

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Introduction

The Echinoderm microtubule-associated protein-like 4 and the anaplastic lymphoma kinase (EML4-ALK) fusion genes were discovered in non-small cell lung cancer (NSCLC) in 2007 [1]. The EML4-ALK gene is a fusion gene from a chromosome rearrangement between the N-terminal portion of the echinoderm microtubule associated protein-like 4 (EML4) gene and the tyrosine kinase (TK) domain of the anaplastic lymphoma kinase (ALK) gene, both located on the short arm of chromosome 2, leading to a chimeric oncoprotein with constitutive TK activity and oncogenic transforming activity. Multiple EML4-ALK fusion variants have been identified. The truncations of EML4 may occur at different exons (2, 6, 13, 14, 15, 17, 18 and 20), and the TK domain of the ALK gene begins in exon 20 [2–5]. In addition to EML4-ALK, other ALK fusions have also been reported in lung cancer, including TFG-ALK [6], KIF5B-ALK [7] and KLC1-ALK [8]. Crizotinib, an orally available small-molecule tyrosine kinase inhibitor (TKI) targeting ALK, ROS1 and MET, is highly effective in patients with ALK-positive NSCLC with an objective response rate of approximately 60% [9]. The U.S. Food and Drug Administration (FDA), the European Medicines Evaluation Agency (EMEA), and the China Food and Drug Administration (CFDA) have granted full approval to crizotinib for the treatment of patients with ALK-positive NSCLC.

The incidence of the ALK translocation in NSCLC has been reported to be approximately 3–13% in the Western and Chinese populations [10–17]. The ALK fusion genes appear to be more common in younger patients, patients who were never or light
smokers, and patients with adenocarcinoma [17]. Some studies have reported that ALK rearrangements were not associated with smoking status [11,14,15]. In general, the association between the ALK rearrangements and the patient demographics varied depending on the population studied and the screening methods used.

Currently, the three primary methods of detecting ALK rearrangements are fluorescent in situ hybridization (FISH), immunohistochemistry (IHC), and the reverse transcriptase polymerase chain reaction (RT-PCR). Each of these individual methods has both advantages and disadvantages. FISH has been considered the gold standard method for detecting ALK rearrangements, as it can detect rearrangements irrespective of the EML4-ALK gene fusion variants and other fusion partners. However, FISH is expensive, generally requires specialized technical resources and expertise and thus cannot be applied in all pathological laboratories and is unavailable for screening in daily practice. Several antibodies have been investigated for detecting ALK rearrangements. Because the expression level of the fusion protein is lower in NSCLC than in anaplastic lymphoma, the development of routine IHC has been problematic [18–20]. IHC requires the standardization of reagents and protocols across pathology laboratories. The Ventana ALK assay is a new method of detecting ALK rearrangements that uses D5F3 antibody and relies on the tyramide amplification technique bound to the Ventana automated BenchMark XT for high sensitivity. Several studies have demonstrated that there is a high concordance between the Ventana IHC and the FISH [21,22]. The RT-PCR is a more sensitive and specific method that can identify variants of the ALK rearrangements, but all possible ALK translocations with EML4 and the other fusion partners must be accounted for in the primer design to detect them. Therefore, detecting ALK rearrangements continues to be challenging. Only FISH using a break-apart probe kit (Vysis LSI ALK Dual Color, break-apart rearrangement probe; Abbott Molecular, Abbott Park, IL) is approved by the FDA for the treatment of proven ALK-positive NSCLC. However, other detection methods may be approved in the future.

In this study, the ALK rearrangements in patients with primary lung adenocarcinoma were investigated, and results obtained through FISH, Ventana IHC, and RT-PCR were compared. The frequency of the ALK rearrangements in a group of randomly selected hospitalized patients from Beijing, China, was determined, and its association with clinicopathological characteristics and outcomes were further explored.

**Materials and Methods**

**Patients**

This study enrolled patients were hospitalized between 2005 and 2013 at Beijing Chest Hospital, Beijing, China. Their clinicopathological data included age, gender, smoking status, tumor, node, metastases (TNM) stage, treatment history, and follow-up. All patients had sufficient tissue for a tissue microarray. Non-smokers were defined as patients who had smoked <100 cigarettes in their lifetime. The TNM staging was reviewed according to the 7th edition of the American Joint Committee for Cancer [AJCC] staging system [23]. The histological subtypes of the adenocarcinomas were classified according to the new International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) multidisciplinary classification of lung adenocarcinoma [24]. The Response Evaluation Criteria in Solid Tumors (RECIST) [25] was used, including complete response (CR), partial complete (PR), stable disease (SD), and progression of disease (PD). The overall response rate (ORR) contains the CR and the PR. The progression-free survival (PFS) time was measured from the first date of treatment until the date of the first documented disease progression or until the date of death for any reason in the absence of disease progression. The overall survival (OS) in this study was measured from the date of the first operation for patients who underwent surgery or of first-line anti-tumor therapy for advanced patients until the date of death for any reason. The present study was conducted according to the principles of the Declaration of Helsinki and approved by the ethical committees of Beijing Chest Hospital. All of these patients assigned written informed consent, and the ethics committees approved the consent procedure.

**Specimen preparation**

A slide was cut from the formalin-fixed, paraffin-embedded (FFPE) blocks of selected patients with primary lung adenocarcinoma for hematoxylin and eosin staining. The samples containing more than 75% tumor cells were enrolled. Other three- to five-μm-thick slides were cut from the blocks and placed into two Eppendorf tubes for the DNA extraction and the RNA extraction. Three cores of the tissue, each with a diameter of 2 mm from the tumor areas marked by the pathologists, were patched from the block for each patient and were used for tissue microarrays (TMA). The EGF status of each of the patients was identified by the DNA sequencing method. Serial sections of the TMAs were cut, and hematoxylin and eosin staining, FISH, and IHC were performed.

**Detection of ALK rearrangements**

FISH was performed on the 4-μm-thick slides of FFPE TMA using the Vysis ALK Break Apart FISH Probe Kit (Vysis LSI ALK Dual Color, break-apart rearrangement probe; Abbott Molecular, Abbott Park, IL) according to the manufacturer’s instructions. At least 100 representative tumor cells were counted, and the occurrence of an ALK gene rearrangement was concluded if ≥15% of the tumor cells showed a split red and green signal and/or an isolated (single) red signal. Otherwise, the specimen was classified as ALK FISH negative. The results obtained by FISH were analyzed using an Olympus fluorescence microscope equipped with orange, green, and 4’, 6-diamidino-2-phenylindole filters. Images were captured using the Video Test Image Analysis System.

IHC was performed on the 4-μm-thick slides of FFPE TMA on a Benchmark XT stainer. The pre-diluted Ventana anti-ALK (D5F3) rabbit monoclonal primary antibody (Cell Signal Technology, U.S.) was applied, and the Optiview DAB IHC detection kit and the Optiview Amplification kits were used according to the manufacturers’ instructions. Each patient sample included a matched rabbit monoclonal IgG negative control. The scoring algorithm for the ALK IHC was that the presence of strong granular cytoplasmic staining in the tumor cells (any percentage of positive tumor cells) were concluded to be positive for ALK, while the absence of strong cytoplasmic staining in the tumor cells indicated that they were negative for ALK.

The ALK rearrangements were tested by RT-PCR using an AmoyDx ALK Fusion Gene Detection Kit (Amoy Diagnostics, Xiamen, China). The positive PCR products were verified by sequencing on an ABI 3500xl at Amoy Diagnostics, China. The primers sequences used on sequencing are followed: EML4-ALK variant 1: F: GGAGGCAAAAACTACGTAGAGCCC; M13R-R: AGGGGATACAATTTTCCAAGGAGCTTG-CAGCTCCTGGTGCT; EML4-ALK variant 2: F: ACAAGTA-TATAATGTTCAACTGCGGAG; M13R-R: AGGGGATACAAATTTTCCAAGGAGGGCTTG-CAGCTCCTGGTGCT; EML4-ALK variant 3a/b: F: ACTGCAGACAGCCTGGTGCT;
GATGTC, M13R-R: AGCGGATAACAATTTTCACACAGGACATGGCAGCTCCTGGTGCT; KIF5B-ALK: F: AGTAGATCGCATAAGGAGGGAGTC, M13R-R: AGCGGATAACAATTTTCACACAGGACATGGCAGCTCCTGGTGCT.

Statistical analysis

Fisher’s exact test was used to examine the association between the ALK rearrangements and the clinicopathological factors. The continuous data were analyzed by the Kruskal-Wallis test. The ORR was compared by Fisher’s exact test between the different groups. The Kaplan-Meier method was used to estimate the PFS or OS between the different groups. The Cox proportional hazards regression model was used to identify independent factors of OS. All of the statistical tests were performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL). The P values were 2 tailed for all of the tests. Statistical significance was set at P<0.05.

Results

Patients

A total of 430 patients with primary lung adenocarcinoma were available for analysis. The clinicopathological characteristics of all of the patients with lung adenocarcinoma are listed in Table 1.

ALK rearrangements

Forty-six (10.7%) of the patients were identified as ALK-positive by FISH, of which one patient had a co-mutation with the ALK rearrangement and EGFR mutation (L858R), and 199 (46.3%) had the wild type for both the ALK and the EGFR (defined as WT/WT). The association between the genotypes and clinicopathological characteristics are shown in Table 2.

Compared with the patients with the EGFR mutations, the ALK-positive patients were significantly younger than the patients harboring the EGFR mutations, with a median age of 52 compared with 57 (P<0.001), respectively. The incidence of ALK rearrangements in the solid subtype was significantly higher than in the acinar subtype and the papillary subtype (P=0.044, P<0.001); the incidence of the ALK rearrangements in the acinar subtype was significantly higher than in the papillary subtype (P=0.011). There were no differences in the incidence of the ALK rearrangements and EGFR mutations for gender, smoking status, or stage. Compared with the WT/WT patients, the ALK-positive patients were significantly younger than the patients harboring the WT/WT genotype, with a median age of 52 compared with 60 (P<0.001), respectively. There were no differences in the gender, smoking status, stage, or histological subtype between the ALK-positive and the WT/WT patients. The incidence of the ALK rearrangements in the patients harboring the EGFR wild type was significantly higher than in the EGFR mutations (45/244, 18.4% versus 1/186, 0.5%, P<0.001). Images of the results of the FISH are shown in Figure 1.

The IHC showed that 53 (12.3%) patients were ALK-positive with a strong granular cytoplasmic staining in the tumor cells (Figure 2). The incidence of the ALK rearrangements by the IHC in patients harboring the EGFR wild type was significantly higher than in the patients with the EGFR mutations (52/244, 21.5%, 1/186, 0.5%; P<0.001).

RT-PCR was performed for 200 patients, including 46 with ALK-positive and 154 with ALK-negative results detected by FISH. Sixty-four patients were positive for the ALK rearrangements (32.0%). All of the ALK-positive specimens were confirmed by direct sequencing. Among the ALK-positive patients, 62 (96.9%) had EML4-ALK fusion genes, and 2 (3.1%) had KIF5B-ALK fusion genes in which exon 24 of the KIF5B and exon 20 of the ALK were jointed. The variant fusion types were identified from the 62 patients with the EML4-ALK fusion genes. These included variant 1 (E13:A20) in 24 patients (38.7%), variant 2 (E20:A20) in eight patients (12.9%), and variant 3a/b (E6a/b:A20) in 30 patients (48.4%). The two patients harboring KIF5B-ALK fusion gene had no EGFR mutations and belonged to the acinar subtype. Both of them were non-smoking males, one of whom was 48 years old with stage IIIA and died 34.5 months after surgery and the other of whom was 55 years old with stage IV and died 6 months after diagnosis.

Of the 200 patients detected by RT-PCR, 84 patients had EGFR mutations and 116 patients had the wild type. The incidence of the ALK rearrangements in the patients harboring the EGFR wild type was significantly higher than in the patients with the EGFR mutations (55/116, 47.5%, 11/84, 13.1%; P<0.001). The details of the 11 patients who had co-mutations with the ALK rearrangement and the EGFR mutation are shown in Table 3.

Comparison of the ALK rearrangements detection among FISH, Ventana IHC, and RT-PCR

In the correlation analysis of these three methods, FISH was considered to be the gold standard method. Of the 430 patients detected by FISH and IHC, 46 patients were both FISH and IHC positive, 7 patients were IHC positive/FISH negative, and 377 patients were both FISH and IHC negative. The IHC sensitivity and specificity were 100% and 98.2%, respectively. The concordance rate between the FISH and the IHC for detecting the ALK rearrangements was 98.4%.

Of the 200 patients detected by FISH, IHC and RT-PCR, 44 of the 46 FISH positive patients were also IHC positive/RT-PCR positive. Two of the FISH positive/IHC positive patients were negative on the RT-PCR. Of the 7 FISH negative/IHC positive patients, 6 patients were positive on the RT-PCR, and 1 patient was RT-PCR negative. Six of the 20 patients who were FISH negative/RT-PCR positive were IHC positive, and 14 patients were IHC negative. One hundred and thirty-three patients were FISH/IHC/RT-PCR negative. Two patients harboring the KIF5B-ALK fusion gene were also determined to be ALK positive by FISH and IHC. The sensitivity and specificity of the RT-PCR were 95.7% and 87.0%, respectively. The concordance rate of the FISH and the RT-PCR was 89.0% (Table 4).

The outcomes

Of the 430 patients, 216 patients with recurrent or advanced disease who received systemic treatment were available for an analysis of its efficacy. For an analysis of the efficacy, the patients were divided into three groups consisting of the ALK rearrangements, the EGFR activating mutations (exon 19 deletions and exon 21 mutation), and the WT/WT for both the ALK rearrangements and EGFR wild types. Of the 216 patients, 171 patients with recurrences or advanced patients received chemotherapy as the first-line treatment and 74 patients received TKIs as the first-line treatment. Among the 171 patients, 22 had ALK rearrangements, 62 had EGFR activating mutations, and 87 had the WT/WT. The best response to the first-line chemotherapy was analyzed. The ORR of the chemotherapy for patients with ALK rearrangements, EGFR activating mutations, and WT/WT were 31.8%, 37.1%, and 25.3%, respectively. The PFS of the first-line chemotherapy for patients harboring ALK rearrangements, EGFR activating mutations, and WT/WT were 3.8 months, 4.5 months, and 3.5 months, respectively. There were no significant differences in the ORR and PFS among the three groups in the first-line
chemotherapy (Figure 3A). Among the 171 patients, 46 patients received pemetrexed combined platinum or pemetrexed mono-therapy. Of the 46 patients, three patients were ALK-positive, of which patients received pemetrexed treatment as the first line. The response rate and PFS were SD and 7.3 months and PR and 12 months, respectively. The third ALK-positive patient received pemetrexed treatment in the second line had SD and the PFS was 6 months. A statistical analysis was not conducted due to the small sample size of the patients received pemetrexed.

Of the 216 patients, 97 patients who received the EGFR TKIs treatment were available for an analysis of the response, including 45 patients in the first line, 43 patients in the second line, and nine patients in the third line. Among the patients receiving the EGFR TKIs, 10 had ALK rearrangements, 47 had EGFR activating mutations, and 40 had the WT/WT. The best response to the EGFR TKIs in all of the lines was analyzed. The ORR of EGFR TKIs for patients harboring ALK rearrangements, EGFR activating mutations, and the WT/WT were 0.0%, 68.1%, and 12.5%, respectively. The ORR for the patients harboring the EGFR activating mutations was significantly higher than for the patients harboring the ALK rearrangements and the WT/WT (P<0.001, P<0.001, respectively). The PFS for the patients harboring the ALK rearrangements, EGFR activating mutations, and WT/WT were 1.3 months, 11.0 months, and 2.0 months, respectively. The PFS for the patients harboring the EGFR activating mutations was significantly higher than for the patients harboring the ALK rearrangements or WT/WT (P<0.001, P<0.001, respectively) (Figure 3B). No significant differences in the ORR and PFS between the ALK rearrangements and the WT/WT group were observed. The efficacy for the patients with different genotypes receiving chemotherapy or EGFR TKIs is shown in Table 5.

The latest follow-up was performed on 30th, September 2013. Of the 299 patients, 94 patients had died, 94 patients were still alive, and 37 patients failed to follow-up. The overall survival was analyzed in the patients with early stage and advanced disease. The patients who were still alive or failed to follow-up were regarded as censors in the statistical analysis.

Of the 299 patients, the median OS was 18.7 months (95% CI 16.892–20.508). The OS for the patients who harboring ALK rearrangements, EGFR activating mutations, and WT/WT were

### Table 1. Characteristics of all patients.

| Characteristics                  | N (%) |
|---------------------------------|-------|
| Age (year)                      |       |
| Range                           | 23–82 |
| Median                          | 57    |
| Gender                          |       |
| Male                            | 229 (53.3) |
| Female                          | 201 (46.7) |
| Smoking status                  |       |
| Non-smoking                     | 263 (61.2) |
| Smoking                         | 167 (38.8) |
| Stage                           |       |
| I                               | 78 (18.1) |
| II                              | 28 (6.5) |
| IIIA                            | 114 (26.5) |
| IIIB+IV                         | 201 (46.7) |
| Stage unknown                   | 9 (2.1) |
| Histologic subtype              |       |
| Lepidic predominant             | 12 (2.8) |
| Acinar predominant              | 236 (54.9) |
| Papillary predominant           | 101 (23.5) |
| Micropapillary predominant      | 20 (4.7) |
| Solid predominant with mucin production | 51 (11.9) |
| Invasive mucinous adenocarcinoma| 7 (1.6) |
| Colloid variant                 | 3 (0.7) |
| EGFR status                     |       |
| Mutation                        | 186 (43.3) |
| Exon 18 mutation                | 9 (2.1) |
| Exon19 mutation                 | 94 (21.9) |
| Exon 20 mutation                | 3 (0.7) |
| Exon 21 mutation                | 77 (17.9) |
| Multiple mutation               | 3 (0.7) |
| Wild type                       | 244 (56.7) |

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Table 2. Association of different genotypes with clinicopathological characteristics in 430 patients (ALK rearrangement results based on FISH detection).

|                          | ALK rearrangement (n = 46) | EGFR mutation (n = 186) | WT/WT (n = 199) | P (ALK vs. EGFR) | P (ALK vs. WT/WT) |
|--------------------------|---------------------------|-------------------------|-----------------|------------------|------------------|
|                          | n  | %   | n  | %   | n  | %   |                 |                 |
| Age (years old)          |    |     |    |     |    |     |                 |                 |
| Median                   | 52 | 54.3 | 57 | 41.4 | 60 | 30.7 | <0.001          | <0.001          |
| Range                    | 23–69 | 28–79 | 26–82 |                 |                 |
| Gender                   |    |     |    |     |    |     |                 |                 |
| Male                     | 25 | 54.3 | 77 | 41.4 | 128 | 64.3 | 0.136           | 0.238           |
| Female                   | 21 | 45.7 | 109 | 58.6 | 71 | 35.7 |                 |                 |
| Smoking status           |    |     |    |     |    |     |                 |                 |
| Non-smoking              | 29 | 63.0 | 136 | 73.1 | 98 | 49.2 | 0.204           | 0.103           |
| Smoking                  | 17 | 37.0 | 50 | 26.9 | 101 | 50.8 |                 |                 |
| Stage                    |    |     |    |     |    |     |                 |                 |
| I                        | 6  | 13.0 | 41 | 22.0 | 31 | 15.6 | 0.129           | 0.129           |
| II                       | 0  | 0.0  | 9  | 4.8  | 19 | 9.5  |                 |                 |
| IIIA                     | 12 | 26.1 | 53 | 28.5 | 50 | 25.1 |                 |                 |
| IIIB+IV                  | 28 | 60.9 | 78 | 41.9 | 95 | 47.7 |                 |                 |
| Unknown stage            | 0  | 0.0  | 5  | 2.7  | 4  | 2.0  |                 |                 |
| Histologic subtype*      |    |     |    |     |    |     |                 |                 |
| Lepidic predominant      | 0  | 0.0  | 7  | 3.8  | 5  | 2.5  | <0.001          | 0.074           |
| Acinar predominant       | 27 | 58.7 | 99 | 53.2 | 110 | 55.3 |                 |                 |
| Papillary predominant    | 4  | 8.7  | 58 | 31.2 | 39 | 19.6 |                 |                 |
| Micropapillary predominant| 3  | 6.5  | 15 | 8.1  | 26 | 13.1 |                 |                 |
| Solid predominant        | 11 | 23.9 | 15 | 8.1  | 26 | 13.1 |                 |                 |
| Invasive mucinous adenocarcinoma | 0 | 0.0 | 0 | 0.0 | 7 | 3.5 |                 |                 |
| Colloid variant          | 1  | 2.2  | 0  | 0.0  | 2  | 1.0  |                 |                 |

*One patient had a co-mutation of EGFR and ALK.

*P value was analysis on the frequency of ALK rearrangement among acinar predominant subtype, papillary predominant subtype, and solid predominant subtypes because of small samples of other subtypes.

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13.5 (95% CI 9.599–17.401), 24.2 (95% CI 19.869–28.531), and 15.0 (95% CI 12.206–17.794) months, respectively. The OS for the patients harboring EGFR activating mutations was significantly longer than for the patients harboring ALK rearrangements and WT/WT ($P<0.001$, $P<0.001$). There was no significant difference in the OS between the ALK-positive patients and the WT/ WT patients ($P=0.123$) (Figure 3C).

Factors including the age, gender, smoking status, stage, histological subtypes, EGFR status, ALK status, and EGFR TKIs treatments were analyzed for their association with the OS. Due to the small number of patients with unknown stage, patients with lepidic, micropapillary, invasive mucinous adenocarcinoma and colloid variants were not enrolled in the analysis. The univariate analysis showed that the stage (31.2 months for stage I-IIIA vs. 10.7 months for stage IIIB-IV, $P<0.001$), histological subtypes (15.3 months for the acinar subtype, 24.2 months for the papillary subtype, 17.3 months for the solid subtype, $P<0.001$), EGFR status (24.2 months for the activating mutations vs. 14.3 months for the wild type, $P<0.001$), and ALK status (20.0 months for the ALK-negative vs. 13.0 months for the ALK-positive, $P=0.001$) were associated with the OS. Age (18.9 months for <57 years old vs. 18.0 months for ≥57 years old, $P=0.301$), gender (18.2 months for male vs. 18.9 months for female, $P=0.632$), smoking status (18.9 months for non-smoking vs. 18.2 months for smoking, $P=0.612$), and EGFR TKIs treatment (20.5 months for EGFR TKIs treatment vs. 18.0 months without TKIs treatment, $P=0.589$) were not associated with the OS. The multivariate analysis was performed using a Cox regression model. The Cox analysis showed that an early stage ($P<0.001$, HR 3.707, 95% CI 2.841–4.836) and EGFR activating mutations ($P<0.001$, HR 1.697, 95% CI 1.306–2.205) were independently associated with a longer OS, whereas the histological subtypes ($P=0.129$, HR 0.786, 95% CI 0.539–1.147) (Table 6).

**Discussion**

The ALK rearrangements were screened in this study in a large randomly selected cohort of patients with lung adenocarcinoma by FISH, IHC, and RT-PCR. According to the FISH results, ALK-positive patients were associated with a younger age and with the EGFR wild type rather than with other clinicopathological factors. The incidence of ALK rearrangements was 10.7% (46/430) as obtained by FISH in this study, which was similar to the results previously published [13,14]. We also found that the ALK fusion genes are associated with a younger age and the EGFR wild type, which was consistent with previous studies [13,17,26]. However, we did not find that the ALK rearrangements were associated with non-smoking. This was similar to some of the previous work [11,13,16,26,27,28], but differed from others [17,29], which might be due to the difference in the population studied. Previous studies reported that ALK rearrangements were common in signet cell carcinoma and mucinous adenocarcinoma [19]. According to the new classification of lung adenocarcinoma by the IASLC/ATS/ERS, there were no associations between the ALK rearrangements and histological subtypes.

Previous studies have shown that the Ventana IHC has a high sensitivity and specificity (>98%), as well as good concordance with FISH [21,22]. In our study, the Ventana IHC was performed in all of the 430 patients, with a sensitivity and specificity of 100% and 98.2%, respectively. It demonstrated a high concordance rate of 98.4%, which was similar to the results obtained from the above studies. In addition to its high coherence with FISH, Ventana IHC is quicker, less expensive, easier to control, and has a good repeatability, and it has been approved by the EMEA and CFDA as an aid in identifying patients who are eligible for treatment with crizotinib.

RT-PCR is a highly sensitive method demonstrated in this study. It had a positive rate of 32.0% (64/200) for the detection of ALK rearrangements in 200 patients, which was the highest compared with 23.0% (46/200) and 26.5% (53/200) detected by
### Table 3. Details of 11 patients with a co-mutation of EGFR and ALK by RT-PCR.

| Case | Age | Gender | Smoking status | Stage | Histologic subtype | EGFR Status | Mutation type of EGFR | FISH | Ventana IHC | RT-PCR | Variants or partners |
|------|-----|--------|----------------|-------|--------------------|-------------|----------------------|------|-------------|--------|---------------------|
| 1    | 57  | Female | Non-smoking    | IIIA  | Acinar predominant | Mutation    | 19 deletion          | Negative | Negative | Positive | v3a/b               |
| 2    | 55  | Female | Non-smoking    | IV    | Acinar predominant | Mutation    | 19 deletion          | Negative | Negative | Positive | v3a/b               |
| 3    | 49  | Male   | Smoking        | IV    | Acinar predominant | Mutation    | 19 deletion          | Negative | Negative | Positive | v1                  |
| 4    | 45  | Male   | Non-smoking    | IV    | Acinar predominant | Mutation    | 19 deletion          | Negative | Negative | Positive | v1                  |
| 5    | 34  | Female | Non-smoking    | IV    | Acinar predominant | Mutation    | 19 deletion          | Negative | Negative | Positive | v3a/b               |
| 6    | 34  | Female | Non-smoking    | I     | Micropapillary predominant | Mutation | 19 deletion | Negative | Negative | Positive | v1                  |
| 7    | 46  | Male   | Smoking        | I     | Papillary predominant | Mutation | 19 deletion | Negative | Negative | Positive | v1                  |
| 8    | 58  | Male   | Smoking        | IIIA  | Solid predominant | Mutation    | L858R                | Positive | Positive | Positive | v3a/b               |
| 9    | 63  | Female | Non-smoking    | I     | Papillary predominant | Mutation | 19 deletion | Negative | Negative | Positive | v3a/b               |
| 10   | 56  | Female | Non-smoking    | IV    | Acinar predominant | Mutation    | 19 deletion          | Negative | Negative | Positive | v3a/b               |
| 11   | 60  | Male   | Smoking        | I     | Papillary predominant | Mutation | 19 deletion | Negative | Negative | Positive | v1                  |

### Table 4. Comparison of FISH and IHC in 430 patients and comparison of FISH and RT-PCR on detecting ALK rearrangements among 200 patients.

|                | FISH Total | Sensitivity | Specificity | Concordance rate |
|----------------|------------|-------------|-------------|------------------|
|                 | Positive   | Negative    |             |                  |
| Ventana IHC     | 46         | 7           | 53          | 100%             | 98.2%         | 98.4%         |
| RT-PCR          | 44         | 20          | 64          | 95.7%            | 87.0%         | 89.0%         |

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FISH and IHC, respectively, which was further confirmed by sequencing. RT-PCR can also identify variants or different fusion partners. In our study, variant 1 (38.7%) and variant 3a/b (48.4%) were the common variants, and variant 2 was less common, which is similar to a previous study reporting that the most common variants were variant 1 (33%) and variant 3a/b (29%) [1]. However, the frequency was different, which may be due to the population studied. The exact frequency of the variants and their clinical significance remain under investigation. Different EML4-ALK variants may exhibit different sensitivities to crizotinib [30]. KIF5B-ALK was detected in two cases with variants of KIF5B-ALK in which exon 24 of KIF5B and exon 20 of ALK were joined. FISH and IHC also detected the KIF5B-ALK fusion gene in these two patients, which demonstrated that both methods are independent of fusion partners. The RNA quantity and quality are critical for the detection of the ALK rearrangement using RT-PCR. RNA degradation of the FFPE may occur, which induces false negative results. Two patients who were FISH and IHC positive but were negative on the RT-PCR may be explained by the RNA degradation in the FFPE tissue samples. The other explanation may be related to RT-PCR, which can only detect the known rearrangements. As RT-PCR is a PCR-based method, the sample contamination issue should be considered in the process of cutting the slides from the tissue and in the preparation of the PCR reaction mix. The sequencing of the PCR product following the RT-PCR procedure is a good method to ensure the accuracy of the result.

In this study, the RT-PCR revealed a sensitivity and specificity of 95.7% and 87.0%, respectively, and a concordance rate of 89.0% between the FISH and the RT-PCR, which was slightly lower than in a previous study [21]. We had a higher frequency of cases that they were RT-PCR positive but FISH negative. This may be related to the differences in the sample types collected from the patients enrolled. The majority of the samples in this study were from resected tumors or lymph nodes rather than a small biopsy. These types of samples enabled the acquisition of a higher quality and quantity of RNA and improved the positive rates. There are also several reasons for the discordance between the FISH and RT-PCR: first, splitting the red and green FISH signals can be extremely subtle, which leads to false-negative results, and a small proportion of atypical results from an ALK FISH interpretation may be discrepant; second, the cross-contamination of RT-PCR samples and non-specific amplification may also cause false positive results. In our study, the positive ALK rearrangement observed on the RT-PCR in patients with the EGFR wild type was 45.7% (53/116), which was higher than the result of 34% reported in a previous study [27]. The reason for these varied results may be related to the population and to the tissue differences. The correlation between FISH and RT-PCR needs to be demonstrated further in a large sample population.

Using FISH or IHC, one of the 430 (1/186, 0.5%) patients, a 58-year-old male patient with solid subtype had a coexisting of ALK rearrangement and EGFR mutation (L858R in exon 21). On the RT-PCR, the frequency of the concomitant mutation was 13.1% (11/84), which was similar to the 13.8% of the coexistence of ALK and EGFR with RT-PCR [31]. The frequency may be due to the high sensitivity of the RT-PCR. In regard to the reasons for the different frequencies of the concomitant mutations on FISH and RT-PCR, some of which are similar to those noted above, may be due to false negatives on FISH or false positives on RT-PCR.

Figure 3. Progression-free survival (PFS) and overall survival (OS) among ALK-positive patients, patients who have EGFR activating mutations and wild type both ALK and EGFR (WT/WT). (A) PFS for patients receiving the first-line chemotherapy harboring ALK rearrangements, EGFR activating mutations, and WT/WT. (B) PFS for patients receiving EGFR TKIs harboring ALK rearrangements, EGFR activating mutations, and WT/WT. (C) OS for patients harboring ALK rearrangements, EGFR activating mutations, and WT/WT.

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Table 5. Treatment response and PFS according to different genotypes in patients with recurrent or advanced diseases.

|                        | n    | ALK rearrangement | EGFR activating mutation WT/WT | P (ALK vs. EGFR) | P (ALK vs. WT/WT) | P (EGFR vs. WT/WT) |
|------------------------|------|-------------------|-------------------------------|-----------------|------------------|-------------------|
| No. of patients evaluated in first line chemotherapy |      |                   |                               |                 |                  |                   |
| CR                     | 171  | 22                | 62                            | 0.797           | 0.592            | 0.086             |
| PR                     | 22   | 7 (31.8)          | 23                            | 0.001           | 0.569            | <0.001            |
| SD                     | 62   | 12 (54.5)         | 31                            | 0.001           | 0.569            | <0.001            |
| PD                     | 87   | 3 (13.6)          | 8                             | 0.001           | 0.569            | <0.001            |
| ORR                    |      |                   |                               |                 |                  |                   |
| PFS, month (95% CI)    |      |                   |                               |                 |                  |                   |
| No. of patients evaluated in any-line TKIs therapy | 97   | 10                | 47                            | 1.3 (0.215–2.385)| 0.801            | 0.407             |
| CR                     | 0 (0.0) | 2 (4.3)           | 0 (0.0)                       | 0.801           | 0.407            | 0.041             |
| PR                     | 0 (0.0) | 0 (0.0)           | 30 (63.8)                     | 0.801           | 0.407            | 0.041             |
| SD                     | 3 (30.0) | 3 (6.4)           | 12 (25.5)                     | 0.801           | 0.407            | 0.041             |
| PD                     | 7 (70.0) | 3 (6.4)           | 12 (25.5)                     | 0.801           | 0.407            | 0.041             |
| ORR                    | 0 (0.0) | 0 (0.0)           | 32 (68.1)                     | 0.801           | 0.407            | 0.041             |
| PFS, month (95% CI)    | 1.3 (0.215–2.385) | 11.0 (8.901–13.099) | 2.0 (1.229–2.771) | <0.001 | 0.169 | <0.001 |
PCR. In addition, Wallander [32] reported that cases with EML4-ALK variant 1, as detected by RT-PCR, were hardly detectable by IHC, with either no or faint staining on FISH, as the distance between the two ALK probes was less than two signal distances apart. To date, the benefits of crizotinib for ALK-positive patients are available from clinical trials based on the Vysis break-apart FISH assay. However, to examine the ability of these multiple different ALK detection assays to predict the benefits of crizotinib, well-designed prospective studies including these assays should be constructed in the future.

In the present study, we also analyzed the outcomes based on the genotypes. No differences in the ORR and the PFS for the first-line chemotherapy were observed in patients who were ALK-positive and had EGFR activating mutations and the WT/WT. The response rate and PFS of the EGFR TKIs for any lines in the patients harboring EGFR activating mutations was significantly higher and longer than in patients who were ALK-positive and the WT/WT. These results are quite similar to the studies reported previously [17,26,28]. Previous studies reported that ALK-positive patients have a significantly longer PFS on pemetrexed [33,34]. In our cohort, three ALK-positive patients received pemetrexed treatment, and the PFS for the three patients was 6, 7.3 and 12 months. Due to the very small sample size, the difference in the response to pemetrexed between the ALK-positive and negative patients was not compared.

In the OS analysis, the OS for patients harboring EGFR activating mutations was significantly longer than for patients harboring ALK rearrangements or WT/WT, and there was no difference in the OS between patients harboring ALK rearrangements and WT/WT. These results were also similar to the previous studies [17,28]. For the analysis of the predictors of OS, the univariate analysis showed that the stage, histological subtypes, EGFR status and ALK status were associated with the OS. The Cox analysis demonstrated that an early stage and EGFR activating mutations were independent factors of longer overall survival. However, the ALK status was not an independent factor of OS, which is similar to the finding in a previous study [17].

In conclusion, the ALK rearrangement was studied in a large unselected sample collection of lung adenocarcinoma. An occurrence of 10.7% of ALK rearrangements was identified, which was associated with a younger age and the EGFR wild type. The FISH and the Ventana IHC had a better concordance. RT-PCR is a more sensitive method and can identify different variants or partners. The IHC and RT-PCR need to be further evaluated in clinical trials to identify their roles in guiding patients’ targeted therapy using crizotinib. In an era of targeted therapy, the status of EGFR and ALK should be identified in patients with lung adenocarcinoma before treatment.

### Table 6. Univariate and multivariate analysis for overall survival.

| Variables               | OS (month) | Univariate | Multivariate |
|-------------------------|------------|------------|--------------|
|                         |            | P         | 95% CI       | P           | HR (95% CI) |
| Age (years old)         |            |           |              |             |             |
| <57                     | 18.9       | 0.301     | 16.196–21.604|             |             |
| ≥57                     | 18.0       |           | 15.514–20.486|             |             |
| Gender                  |            |           |              |             |             |
| Male                    | 18.2       | 0.632     | 15.113–21.287|             |             |
| Female                  | 18.9       |           | 16.723–21.077|             |             |
| Smoking status          |            |           |              |             |             |
| Non-smoking             | 18.9       | 0.612     | 16.444–21.356|             |             |
| Smoking                 | 18.2       |           | 15.522–20.878|             |             |
| Stage                   |            |           |              |             |             |
| I+II+IIIA               | 31.2       | <0.001    | 25.134–37.266| <0.001      | 3.707 (2.841–4.836) |
| IIB+IV                  | 10.7       |           | 9.231–12.169 |             |             |
| Histologic subtypes     |            |           |              |             |             |
| Acinar                  | 15.3       | <0.001    | 12.622–17.978| 0.129       | 0.869 (0.725–1.042) |
| Papillary               | 24.2       |           | 16.628–31.772|             |             |
| Solid                   | 17.3       |           | 14.131–20.469|             |             |
| EGFR status             |            |           |              |             |             |
| Activating mutation     | 24.2       | <0.001    | 19.923–28.477| <0.001      | 1.697 (1.306–2.205) |
| Wild type               | 14.3       |           | 12.350–16.250|             |             |
| ALK status              |            |           |              |             |             |
| Positive                | 13.0       | 0.001     | 9.484–16.516 | 0.212       | 0.786 (0.539–1.147) |
| Negative                | 20.0       |           | 17.627–22.373|             |             |
| EGFR TKIs treatment     |            |           |              |             |             |
| With                    | 20.5       | 0.589     | 16.388–24.612|             |             |
| Without                 | 18.0       |           | 15.585–20.415|             |             |

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Author Contributions
Conceived and designed the experiments: JW YC YD. Performed the experiments: JW YC YD LZ DS SW XC. Analyzed the data: JW YC YD

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