The Molecular Detection and Clinical Significance of ALK Rearrangement in Selected Advanced Non-Small Cell Lung Cancer: ALK Expression Provides Insights into ALK Targeted Therapy

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Abstract

Background: This study aimed to elucidate clinical significance of anaplastic lymphoma kinase (ALK) rearrangement in selected advanced non-small cell lung cancer (NSCLC), to compare the application of different ALK detection methods, and especially evaluate a possible association between ALK expression and clinical outcomes in crizotinib-treated patients.

Methods: ALK status was assessed by fluorescent in situ hybridization (FISH), immunohistochemistry (IHC) and quantitative RT-PCR (qRT-PCR) in 173 selected advanced NSCLC patients. Clinicopathologic data, genotype status and survival outcomes were analyzed. Moreover, the association of ALK expression with clinical outcomes was evaluated in ALK FISH-positive crizotinib-treated patients including two patients with concurrent epidermal growth factor receptor (EGFR) mutation.

Results: The positivity detection rate of ALK rearrangement by FISH, IHC and qRT-PCR was 35.5% (59/166), 35.7% (61/171), and 27.9% (34/122), respectively. ALK rearrangement was observed predominantly in young patients, never or light smokers, and adenocarcinomas, especially with signet ring cell features and poor differentiation. Median progression-free survival (PFS) of crizotinib-treated patients was 7.6 months. The overall survival (OS) of these patients was longer compared with that of crizotinib-naive or wild-type cohorts, but there was no significant difference in OS compared with patients with EGFR mutation. ALK expression did not associate with PFS; but, when ALK expression was analyzed as a dichotomous variable, moderate and strong ALK expression had a decreased risk of death (P = 0.026). The two patients with concomitant EGFR and ALK alterations showed difference in ALK expression, response to EGFR and ALK inhibitors, and overall survival.

Conclusions: Selective enrichment according to clinicopathologic features in NSCLC patients could highly improve the positivity detection rate of ALK rearrangement for ALK-targeted therapy. IHC could provide more clues for clinical trial design and therapeutic strategies for ALK-positive NSCLC patients including patients with double genetic aberration of ALK and EGFR.

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Introduction

Progress in molecular techniques provides better identification and understanding of molecular markers that may have prognostic value and can drive therapeutic decision making for non-small cell lung cancer (NSCLC). In the past decade, a subset of NSCLC patients with epidermal growth factor receptor (EGFR) mutation has been attracting much attention because of the high response rates to EGFR tyrosine kinase inhibitors (EGFR-TKIs) [1]. In 2007, a fusion gene of anaplastic lymphoma kinase (ALK) with the echinoderm microtubule-associated protein like 4 (EML4) in NSCLC was first identified by Soda et al. [2], and soon became a novel molecular target for lung cancer treatment. Successful experiences of EGFR targeted therapy have provided a reference model for the fast research progress of ALK rearrangement. Crizotinib (ALK/MET/ROS1 inhibitor) was the first clinically
available agent that showed remarkable antitumor activity in ALK-positive advanced NSCLC patients. Recently, selection of patients with ALK rearrangement for crizotinib treatment has become a standard in the USA, European Union, China, Japan, and other countries. More importantly, other ALK inhibitors were successively entered into clinical trials [3] and promising to mark a new page of genotype-driven drug development for lung cancer.

The frequency of ALK rearrangement ranges from 3% to 7% in unselected NSCLC patients, which could reach to 13% ~ 18%, if the patient population is selected according to specific clinicopathologic characteristics, especially in young, never-or light smokers with adenocarcinoma [4,5,6,7,8,9]. In addition, ALK rearrangement was mutually exclusive with EGFR and KRAS mutations. However, above-mentioned characteristics are not shared by all ALK rearrangement carriers. ALK fusion has also been found in older patients, smokers [4], patients with EGFR mutation [10,11,12] and non-adenocarcinoma histological subtypes, such as adenosquamous carcinoma and large cell carcinoma [3,13]. Therefore, clinicopathologic characteristics are insufficient for screening patients and molecular testing is necessary to determine ALK status [14].

Quantitative real-time polymerase chain reaction (qRT-PCR) and fluorescence in situ hybridization (FISH) are the current methods of choice for ALK testing. However, each method has specific advantages and disadvantages. There is no accepted consensus on which method is preferable. qRT-PCR can detect ALK rearrangement at mRNA level and define both ALK fusion partner and fusion variant, but it needs high quality of RNA and cannot detect unknown ALK rearrangements. In addition, there are a number of EML4-ALK variants and non-EML4-ALK fusions in NSCLC [15]. Therefore, qRT-PCR is not widely in use in the detection of ALK rearrangement. FISH is the current standard method to detect ALK rearrangement, since it can detect inversion and translocation irrespective of EML4-ALK gene fusion variants and other fusion partners. Importantly, all clinical trials which showed the effectiveness of crizotinib for ALK-positive NSCLC patients were based on the Vysis/Abbott ALK break-apart FISH assay. However, FISH is expensive, time-consuming and difficult to interpret. So FISH may not be practical for screening every NSCLC patient. IHC is faster, more economical and widely available. Furthermore, IHC with new antibodies and modified protocols has extended its serviceable range for ALK testing. Several published recommendations [16,17] suggested that ALK FISH analysis can be performed only in IHC-positive cases. However, standard IHC protocols and scoring criteria are lacking, and the correlation between ALK expression and clinical outcomes have not been confirmed to verify the accuracy of IHC.

Therefore, this study collected specimens at the beginning of screening patients for crizotinib clinical trials (PROFILE 1005 or PROFILE 1014) and evaluated ALK status using FISH, IHC and qRT-PCR. Clinicopathologic characteristics and clinical outcomes according to the genotype-specific and therapeutic regimens were analyzed, aiming to elucidate the clinical significance of ALK rearrangement in selected advanced NSCLC patients. Furthermore, we compared the application of different ALK detection methods and especially evaluated a possible association between ALK expression and clinical outcomes in ALK FISH-positive crizotinib-treated patients.

**Materials and Methods**

**Study Population and Data Collection**

Specimens were collected from 173 advanced nonsquamous NSCLC patients who were aiming at undergoing ALK screening for crizotinib clinical trials (PROFILE 1005 or PROFILE 1014) from January 2011 to October 2012. All patients received treatment or consultation from Cancer Institute and Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College and signed informed consent for future molecular analysis. This study was approved by the Institutional Review Boards of the Chinese Academy of Medical Sciences Cancer Institute and Hospital.

Medical records of all patients were reviewed to collect demographic, clinical and pathologic information. Histology was reviewed based on the criteria of the World Health Organization Classification of lung tumors [18] and the IASLC/ATS/ERS multidisciplinary classification of lung adenocarcinoma [19]. We recorded EGFR mutation status of patients, which had been determined using a bidirectional sequencing method of EGFR exons 18 to 21. We also examined treatment regimens and clinical outcomes. Progression-free survival (PFS) was calculated from the initiation of crizotinib to documented progressive disease (PD) or death from any cause. In order to better elucidate the influences of genotype-specific and therapeutic regimens on patients’ overall survival (OS), two types of OS were analyzed. OS1 and OS2 were respectively defined as the time from initial diagnosis of NSCLC and from signed informed consent to death from any cause. OS1 was comprehensive but more influenced by previous treatments. OS2 was more specific to clarify the effects of crizotinib in treating ALK-positive advanced NSCLC.

**Table 1. Distribution of ALK expression grade and ALK variants according to ALK rearrangement in selected advanced NSCLC patients.**

| ALK (FISH) | ALK expression grade (IHC) | EML4-ALK variants* (qRT-PCR) |
|------------|----------------------------|----------------------------|
|            | 0  | 1+ | 2+ | 3+ | N.A. | NO | EA1 | EA2 | EA3 | N.A. |
| Positive   | 59 | 0  | 5  | 13 | 39  | 2  | 12  | 30  | 1   | 3    | 13  |
| Negative   | 107| 103| 4  | 0  | 0   | 0  | 71  | 0   | 0   | 0    | 36  |
| N.A.       | 7  | 7  | 0  | 0  | 0   | 0  | 5   | 0   | 0   | 0    | 2   |
| Total      | 173| 110| 9  | 13 | 39  | 2  | 88  | 30  | 1   | 3    | 51  |

Abbreviation: No.: number; N.A.: not available; FISH: fluorescence in situ hybridization; IHC: immunohistochemistry; qRT-PCR: quantitative real-time polymerase chain reaction.

*The kit used to detect EML4-ALK variants included nine known variants with three primer mixtures; EA1: variant 1 or 3a/3b; EA2: variant 2 or 4/4’, EA3: variant 5’ or 5a/5b.

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ALK Test

Specimens were tested by IHC, FISH and qRT-PCR. FISH was conducted with the FDA approved ALK probe kit (Vysis LSI ALK dual-color, break-apart rearrangement probe; Abbott Molecular, Abbott Park, IL) and analyzed according to the kit instructions. ALK IHC was performed according to the protocols provided by the antibody (D5F3, Cell Signaling Technology) manufacturer. Similarly to previous studies [20,21], IHC results were scored as 0 when no specific staining was apparent within a tumor; 1+, faint staining intensity in more than 10% tumor cells without any background staining; 2+, moderate staining intensity; 3+, strong staining intensity. The cases with sufficient tissues were extracted total RNA from formalin-fixed paraffin-embedded (FFPE) specimens using RNeasy FFPE kit (Qiagen, Germany). For the detection of EML4-ALK, mRNA was first reverse-transcribed into cDNA and qRT-PCR was performed with a commercial EML4-ALK kit (Amoydx, China) including nine known variants (A20, E13; A20, E6a/6b; A20, E20; A20, E15; A20, E14; A20, E18; A20, E2; A20, E17; A20,) on the ABI 7500 Real-Time PCR System (Applied Biosystems, USA).

Statistical Analysis

Pearson’s chi-square test, Fisher’s exact test or Kruskal-Wallis test was used for statistical analysis of variables, as appropriate. The Kaplan-Meier approach was used to estimate PFS and OS, and the difference between groups was compared by log-rank test. The hazard ratio (HR) was estimated by proportional hazards regression with a 95% Wald confidence interval (95% CI). Data analysis was done with SAS version 9.2, statistical significance was defined as a two-sided $P$-value, $0.05$. Results

ALK Detection Results

One hundred and thirty-two resected and forty one biopsied specimens were analyzed both by IHC and FISH; however, no interpretable IHC results were obtained for two patients and no interpretable FISH results for seven patients. QRT-PCR was successfully performed in 122 specimens. ALK alteration was found in 35.5% (59/166), 35.7% (61/171), and 27.9% (34/122) samples by FISH, IHC, and qRT-PCR, respectively. The details of the detection results according to the three methods are summarized in Table 1.

Patients’ Characteristics and Clinical Outcomes According to Molecular Subtypes

Of the 166 advanced NSCLC patients who were successfully undergone ALK screening by FISH, 59 harbored ALK rearrangement including two (3.4%, 2/59) patients with concurrent EGFR mutation, 20 showed EGFR mutation, 87 were wild type with ALK-negative and EGFR-negative or EGFR-unknown (wild type cohort, WT). The patients most likely to harbor ALK rearrangement were...
young, never or light smokers with poorly differentiated adenocarcinoma, especially with signet ring cell features, comparing with EGFR mutation or wild type cohort (Table 2). The two patients with concomitant ALK rearrangement and EGFR mutation were analyzed separately due to the low incidence rate.

In the 59 patients with FISH-positive ALK rearrangement, 45 received crizotinib in the phase II clinical trial (PROFILE 1005), 8 were enrolled into phase III clinical trial (PROFILE 1014) and 6 did not participate any clinical trial. Because of the crossover effect of pemetrexed and crizotinib in phase III clinical trial, eight patients were excluded for survival analysis. The other 158 patients were grouped into four types: ALK-positive with crizotinib-treated (n = 45), ALK-positive with crizotinib-naive (n = 6), EGFR mutation with TKIs-treated (n = 20) and wild type (n = 87). The baseline features, clinical treatments and outcomes are shown in Table 3. ALK-positive group showed a dramatically younger age distribution than any other group, received more lines of therapy than EGFR mutation group (median, 2.9 vs. 1.8; P = 0.025) and had more patients received pemetrexed chemotherapy than wild-type group (P = 0.020). ALK-positive patients with and without crizotinib treatment had statistically significant difference in both OS1 (median, 39.7 vs. 8.8 months, P = 0.003) and OS2 (median, 22.0 vs. 1.8 months, P<0.001). There was no significant difference between ALK-positive with crizotinib-treated group and EGFR mutation with TKIs-treated group both in OS1 and OS2 (P = 0.249 and P = 0.896, respectively). Additionally, ALK-positive patients with crizotinib-treated had longer OS2 than wild-type patients (median, 32.0 vs. 14.2 months, P = 0.014), but there was no significant difference in OS1 between these two cohorts (39.7 vs. 38.7 moths, P = 0.294) (Figure 1).

Survival Analysis of ALK FISH-positive Crizotinib-Treated Patients

Of the 45 ALK FISH-positive crizotinib-treated patients, 2 had no IHC results, 4 stained 1+, 10 stained 2+ and 29 stained 3+. Of these patients, 30 were identified to have ALK rearrangement by qRT-PCR, 10 showed negative and 5 had no qRT-PCR results. Up to final follow-up, a total of 33 patients (33/45, 73.3%) presented with PD, and 18 patients (18/45, 40.0%) died. The median PFS of the 45 patients was 7.6 months. We analyzed the potential association between ALK expression and clinical outcomes, such as PFS, OS1 and OS2 using univariate analysis and the results are summarized in Table 4. Multivariate analysis was not performed because of the limitation of the small sample size. Increasing ALK expression, as an ordered variable, did not have any association with PFS (HR = 1.08, 0.60–1.95; P = 0.792), OS1 (HR = 0.67, 0.33–1.37; P = 0.275) or OS2 (HR = 0.71, 0.36–1.40; P = 0.325). However, when ALK expression was taken as a dichotomous variable (i.e., IHC scoring 3+/2+ vs. 1+), a IHC score

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**Figure 1. Kaplan-Meier curves of overall survival for advanced NSCLC patients with different genotypes and therapeutic regimens.**

A, B, C, overall survival calculated from diagnosis (OS1); D, E, F, overall survival calculated from signed informed consent at the screening time (OS2). WT: patients with ALK negative and EGFR-negative or EGFR-unknown; | indicates censored cases.

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Table 3. Characteristics of patients for clinical outcome analysis basing on genotype-specific and therapeutic regimens.

| Variables                      | Group A: ALK+ Crizotinib-Treated (n = 45, %) | Group B: ALK+ Crizotinib-Naive (n = 6, %) | Group C: EGFR Mutation with EGFR-TKIs (n = 20, %) | Group D: Wild-Type (n = 87, %) | P value | A vs. B | A vs. C | A vs. D |
|--------------------------------|---------------------------------------------|-----------------------------|---------------------------------------------|--------------------------------|---------|---------|---------|---------|
| Age at diagnosis, years        | Median 47 (25–73)                           | 48 (32–60)                  | 58 (37–72)                                  | 55 (26–77)                     | 0.930   | 0.002   | <0.001  |         |
|                                | Range                                        |                            |                                            |                               |         |         |         |         |
|                                | < 60                                         | 40 (88.9)                   | 5 (83.3)                                    | 12 (60.0)                      | 57 (65.5) | 0.548   | 0.019   | 0.004   |
|                                | ≥ 60                                         | 5 (11.1)                    | 1 (16.7)                                    | 8 (40.0)                       | 30 (34.5) |         |         |         |
| Sex                            | Male                                         | 18 (40.0)                   | 1 (16.7)                                    | 8 (40.0)                       | 45 (51.7) | 0.509   | 1.000   | 0.201   |
|                                | Female                                       | 27 (60.0)                   | 5 (83.3)                                    | 12 (60.0)                      | 42 (48.3) |         |         |         |
| Smoking status                 | Never/light                                   | 37 (82.2)                   | 5 (83.3)                                    | 14 (70.0)                      | 61 (70.1) | 1.000   | 0.436   | 0.132   |
|                                | Heavy                                        | 8 (17.8)                    | 1 (16.7)                                    | 6 (30.0)                       | 26 (29.9) | 0.886   | 0.205   | 0.922   |
| Stage at diagnosis             | Early                                        | 13 (28.9)                   | 1 (16.7)                                    | 9 (45.0)                       | 22 (25.3) |         |         |         |
|                                | Advanced                                      | 32 (71.1)                   | 5 (83.3)                                    | 11 (55.0)                      | 52 (59.8) |         |         |         |
|                                | Unknown                                       | 0 (0)                       | 0 (0)                                        | 0 (0)                          | 13 (14.9) |         |         |         |
| Stage at screening time        | IIIb                                         | 5 (11.1)                    | 0 (0)                                        | 3 (15.0)                       | 8 (9.2)  | 1.000   | 0.375   | 0.966   |
|                                | IV                                           | 40 (88.9)                   | 6 (100)                                     | 17 (85.0)                      | 79 (90.8) |         |         |         |
| Brain metastasis              | No                                           | 32 (71.1)                   | 3 (50.0)                                    | 16 (80.0)                      | 62 (71.3) | 1.000   | 0.452   | 0.580   |
|                                | Yes                                          | 13 (28.9)                   | 2 (33.3)                                    | 4 (20.0)                       | 20 (23.0) |         |         |         |
|                                | N.A.                                         | 0 (0)                       | 1 (16.7)                                    | 0 (0)                          | 5 (5.7)  |         |         |         |
| Bone metastasis               | No                                           | 26 (57.8)                   | 2 (33.3)                                    | 9 (45.0)                       | 50 (57.5) | 0.776   | 0.340   | 0.725   |
|                                | Yes                                          | 19 (42.2)                   | 3 (50.0)                                    | 11 (55.0)                      | 32 (36.8) |         |         |         |
|                                | N.A.                                         | 0 (0)                       | 1 (16.7)                                    | 0 (0)                          | 5 (5.7)  |         |         |         |
| Prior systemic therapies       | Yes                                          | 33 (73.3)                   | 4 (66.7)                                    | 10 (50.0)                      | 45 (51.7) | 0.859   | 0.025   | 0.310   |
|                                | No                                           | 12 (26.7)                   | 2 (33.3)                                    | 10 (50.0)                      | 41 (47.1) | 1.000   | 0.067   | 0.020   |
| Penetrexed at any line         | Median (range)                                | 2.9 (1–7)                   | 3.0 (1–7)                                   | 1.8 (0–6)                      | 2.5 (0–7) |         |         |         |
|                                | Yes                                          | 33 (73.3)                   | 4 (66.7)                                    | 10 (50.0)                      | 45 (51.7) | 0.627   | <0.001  | 0.499   |
|                                | No                                           | 12 (26.7)                   | 2 (33.3)                                    | 10 (50.0)                      | 41 (47.1) |         |         |         |
|                                | N.A.                                         | 0 (0)                       | 0 (0)                                        | 0 (0)                          | 1 (1.2)  |         |         |         |
| TKI at any line               | Yes                                          | 21 (46.7)                   | 4 (66.7)                                    | 20 (100)                       | 46 (52.9) |         |         |         |
ALK Expression and ALK-Targeted Therapy

Rearrangement and EGFR Mutation

During our data collection, two patients harbored coexisting ALK rearrangement and EGFR mutation (one had exon 19 deletion and the other had exon 21 mutation). They shared some clinicopathologic features, including male sex, young age (42 years and 46 years, respectively), adenocarcinoma, and heavy smoking status. Patient 1 was in stage IV and patient 2 was in stage IIIA at diagnosis, and both of them were in stage IV at screening time. In addition, patient 1 was judged inoperable and patient 2 was underwent surgery. Patient 1 received EGFR-TKI (erlotinib) as first-line therapy and the best response was partial remission (PR). Patient 2 received EGFR-TKI (erlotinib) as fourth-line therapy and the best response was stable disease (SD). PFS of erlotinib was 6.2 months (patient 1) and 3.6 months (patient 2). FISH and IHC for ALK status were performed in both patients, and qRT-PCR was just performed in patient 2. Both patients were ALK FISH positive. Patient 1 showed faint ALK expression. Patient 2 had moderate ALK expression (Figure 3) and showed positive by qRT-PCR. Both of them were enrolled into the phase II clinical trial of crizotinib, and PFS of crizotinib was 3.1 months (patient 1) and 21.7 months (patient 2). The OS1 of patient 1 and patient 2 was 20.5 and 48.0 months, respectively. And patient 2 did not present with PD during crizotinib treatment and was still alive at the final follow-up.

Profiles of Patients with Concomitant ALK Rearrangement and EGFR Mutation

In the univariate analysis of clinical factors, patients’ age, sex, smoking status, stages at diagnosis and screening time, brain and bone metastasis at screening time, previous lines of therapy, histology, and type of therapy (including pemetrexed, EGFR-TKIs and radiation therapy) were not associated with clinical outcomes. Eastern Cooperative Oncology Group (ECOG) performance status showed prognostic significance for PFS (HR = 2.06, 1.17–3.61; P = 0.012), OS1 (HR = 2.79, 1.24–6.26; P = 0.013), but no prognostic significance for OS2 (HR = 1.79, 0.86–3.69; P = 0.117).

Discussion

ALK rearrangement has become a clinically important marker in selecting advanced NSCLC patients for molecular targeted therapy. However, the incidence of ALK rearrangement is relatively low, even in the previously reported enriched population. It is difficult to identify the subsets of ALK-positive patients. Therefore, enriching patients with greatest degree and accurate determination of ALK rearrangements are the key importance to screen appropriate candidates for ALK inhibitors. Due to the disadvantages of the current standard FISH, previous comparative studies suggested that IHC, compared with qRT-PCR, was a promising prescreening method for patients with ALK rearrangement. But clinical outcomes data were lacking to understand comprehensively which testing platform was the most accurate to predict response to targeted therapy. Our data may offer new
insights to the molecular detection in identifying appropriate individuals for ALK inhibitors treatment.

ALK is one of the newest tyrosine-kinase targets in NSCLC. It is aberrantly activated due to a chromosomal rearrangement, leading to the expression of an oncogenic fusion kinase, ALK protein. The most common rearrangement subtype is EML4-ALK [15]. In NSCLC, ALK rearrangement is associated with distinct clinicopathologic features, including young age at onset and adenocarcinoma histology in patients with a history of never or light smoking. Generally, the clinicopathologic features of ALK-positive patients in our study were similar to previous studies reported [5,22]. Notably, ALK rearrangement was more common in poor tumor differentiation in our selected population. To date, only Takahashi et al. [23] reported that ALK rearrangement was associated with poor differentiation status in five ALK-positive patients, our data further affirmed this finding in a relatively large sample size.

Approximately one third of patients harbored ALK rearrangement in our study, which was higher than previously reported range. All recruited patients were suggested by the oncologists to screen for ALK targeted therapy based on clinical considerations (adenocarcinoma, advanced stage, young age, non- or light smoking, or previously treated with cytotoxic regimens and/or targeted therapy). The majority of these patients shared some above-mentioned features, and this may actually affect the frequency of positive detection. ALK rearrangement presented a relatively rare incidence rate in unselected population. These data indirectly indicated that the detection rate of ALK gene translocation could be highly improved based on the selective enrichment according to clinicopathologic features. This information could be helpful for screening ALK positive patients with the greatest degree in clinical routine practice.

In absence of ALK targeted agents, the prognostic value of ALK alterations is controversial [24,25,26]. However, with the develop-

Figure 2. Kaplan-Meier curves of clinical outcomes according to ALK expression and qRT-PCR results for ALK FISH-positive crizotinib-treated patients. A, progression-free survival (PFS); B, overall survival calculated from diagnosis (OS1); C, overall survival calculated from signed informed consent at the screening time (OS2). □ indicates censored cases.
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ALK Expression and ALK-Targeted Therapy

Table 4. Univariate analysis for progression-free survival and overall survival of biological and clinical parameters for the 45 ALK-positive crizotinib-treated patients.

| Variables                                      | PFS |        |        |        | OS1 |        |        |        | OS2 |        |
|------------------------------------------------|-----|--------|--------|--------|-----|--------|--------|--------|-----|--------|
| Age at diagnosis (≥60 vs. <60 years)           | 1.21| (0.42–3.46) | 0.723 | 1.25 | (0.28–5.61) | 0.768 | 0.96 | (0.22–4.18) | 0.953 |
| Sex                                            | 0.70| (0.35–1.40) | 0.316 | 0.95 | (0.37–2.47) | 0.920 | 0.86 | (0.33–2.26) | 0.757 |
| ECOG (continuous)                              | 2.06| (1.17–3.61) | 0.012 | 2.79 | (1.24–6.26) | 0.013 | 1.79 | (0.86–3.69) | 0.117 |
| Smoking status (heavy vs. non or light smokers) | 0.91| (0.39–2.32) | 0.910 | 1.87 | (0.66–5.29) | 0.24 | 2.17 | (0.76–6.18) | 0.146 |
| Stage at diagnosis (advanced vs. early stage)  | 1.02| (0.48–2.16) | 0.956 | 2.08 | (0.67–6.44) | 0.205 | 1.54 | (0.51–4.67) | 0.450 |
| Brain metastasis                               | 1.09| (0.33–3.60) | 0.884 | 24.8 | (0.02–26532.93) | 0.367 | 25.1 | (0.05–13501.95) | 0.315 |
| Bone metastasis                                | 1.37| (0.66–2.83) | 0.399 | 0.59 | (0.19–1.80) | 0.351 | 0.75 | (0.25–2.30) | 0.617 |
| Line of therapy (continuous)                   | 1.81| (0.91–3.61) | 0.093 | 1.78 | (0.67–4.68) | 0.245 | 1.92 | (0.76–4.88) | 0.169 |
| Histology (adenoc with SRC features vs. other types) | 0.89| (0.73–1.09) | 0.261 | 0.82 | (0.60–1.12) | 0.216 | 0.98 | (0.74–1.29) | 0.865 |
| Type of therapy (pemetrexed therapy or not)    | 0.91| (0.43–2.56) | 0.907 | 0.81 | (0.23–2.85) | 0.743 | 0.82 | (0.25–3.00) | 0.821 |
| Type of therapy (EGFR-TKIs therapy or not)     | 1.08| (0.54–2.15) | 0.828 | 1.37 | (0.49–3.84) | 0.551 | 2.01 | (0.74–5.49) | 0.171 |
| Type of therapy (radiation therapy or not)     | 1.30| (0.65–2.61) | 0.457 | 0.77 | (0.29–2.05) | 0.596 | 0.95 | (0.36–2.55) | 0.919 |
| IHC scoring (continuous)                       | 1.08| (0.60–1.95) | 0.792 | 0.67 | (0.33–1.37) | 0.275 | 0.71 | (0.36–1.40) | 0.325 |
| IHC scoring (dichotomous) (IHC2+ vs. 1+)       | 0.49| (0.17–1.42) | 0.187 | 0.23 | (0.06–0.84) | 0.026 | 0.35 | (0.10–1.22) | 0.098 |
| IHC scoring (dichotomous) (IHC3+ vs. 2+/1+)    | 1.52| (0.69–3.32) | 0.300 | 0.88 | (0.34–2.30) | 0.792 | 0.84 | (0.32–2.17) | 0.713 |

Abbreviation: PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; Adeno: adenocarcinoma; SRC: signet ring cell. IHC: immunohistochemistry; ECOG, Eastern Cooperative Oncology Group; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; OS1, overall survival calculated from diagnosis; OS2, overall survival calculated from signed informed consent at the screening time.

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opment of molecular targeted agents, such as crizotinib, positive ALK status was a positive predictive marker for ALK inhibitor therapy [27]. We analyzed survival according to molecular status and therapy regimens, and found that crizotinib prolonged OS of ALK-positive patients. Crizotinib-naive patients showed a generally poor outcome, worse than that of the general population of NSCLC patients. Thus, ALK rearrangement is not a favorable prognostic factor in advanced NSCLC. Of note, these results should be interpreted with caution because crizotinib-naive patients with small sample size had worse performance status than that of patients recruited into crizotinib clinical trials. In addition, ALK-positive crizotinib-treated patients had no difference in OS, compared with EGFR mutation TKI-treated patients, although their survival was numerically shorter. This may be due to the difference in the number of prior lines of therapy between the two groups. EGFR-positive patients represent a well established, TKI-sensitive paradigm. Most EGFR-positive patients immediately received EGFR-TKIs treatment after molecular diagnosis and got superior survival. Moreover, crizotinib prolonged ALK-positive patients’ OS calculated from screening time, but did not prolong OS calculated from diagnosis compared with wild-type patients. It was suggested that ALK-positive NSCLC patients might be particularly responsive to pemetrexed chemotherapy [28,29]. Therefore, small events within ALK-positive crizotinib-treated group, patients with unknown EGFR status in wild-type group and different treatment history of pemetrexed between the two groups might have confounded the results.

Although our study had the limitations of a single-center, restricted statistical power because of the small sample size, and confounding factors, including performance status, prior treatment history, unknown EGFR status and small events, we still could clearly reveal the effects of crizotinib in treating ALK-positive advanced NSCLC.

FISH is currently used standard based on the pivotal studies of crizotinib. However, some shortcomings of the ALK FISH assay have been reported. The clinical trials of crizotinib recruited patients who were positive by FISH only, but it was later noted [30] that patients with double-positive of FISH and IHC results had a higher response rate. It was also reported [31] that a patient with ALK IHC-positive and ALK FISH-negative had dramatic response to crizotinib. These findings suggested that there might have been patients with false-positive -negative results by FISH. In addition, the uninformative rate of FISH among tumors reported as IHC-positive was high [32]. Recently, several published recommendations [16,17] suggested that FISH analysis could be performed only in IHC-positive cases and other recommendation [33] indicated that IHC, if carefully validated, may be considered as a screening methodology to select specimens for FISH testing; qRT-PCR was not recommended as an alternative to FISH. But to date, previous studies have not compared the prognostic power of IHC or qRT-PCR. In theory, each biologically relevant ALK rearrangement leads to overexpression of ALK protein, which is the true drug target. In our study, patients with faint staining intensity had inferior clinical
outcomes and poor response to ALK inhibitor. Although multivariate analysis could not be performed due to the small sample size of crizotinib-treated patients, we did demonstrate by univariate analysis that tumor with moderate and strong ALK expression marginally predicted improved OS2 and significantly predicted a decreased risk of death. These findings were unequivocally substantiated by the Kaplan-Meier survival curves, which demonstrated a significantly superior overall survival of patients with moderate or strong ALK expression. The positive association of ALK expression with improved survival is intriguing and surprising. Moreover, ALK positive and negative patients classified by qRT-PCR in crizotinib-treated patients had no significant difference in clinical outcomes. To our knowledge, this is the first report which elucidated the association of ALK expression and qRT-PCR with clinical outcomes to evaluate the application of ALK detection methods. Considering the affordability and sensitivity of IHC and qRT-PCR, an IHC based ALK test may represent a reliable and cost-effective screening strategy in identifying patients who might benefit from ALK inhibitors. Due to the limitation of small sample size, our data support the need for large-scale and prospective biomarker studies to validate this diagnostic strategy for ALK-positive NSCLC.

ALK rearrangement and EGFR mutation are generally mutually exclusive [34]. But the coexistence of ALK and EGFR has been successively reported (>1% of all treated NSCLC) [35,36,37]. In our study, the incidence rate was 3.4%. However, Wang et al. [11] reported a higher frequency (15%) of this double genetic aberration in Chinese patients. It suggested that EGFR mutation and ALK rearrangement may arise independently during oncogenesis and may act synergistically. The two patients harboring the

Figure 3. ALK immunohistochemical staining. A, no staining (score, 0); B, faint staining (score, 1+); C, moderate staining (score, 2+); D, strong staining (score, 3+); E, ALK protein staining of patient with concurrent ALK and EGFR mutation (patient 1, score, 1+); F, ALK protein staining of patient with concurrent ALK and EGFR mutation (patient 2, score, 2+). Magnification, 400×. doi:10.1371/journal.pone.0084501.g003
double genetic aberrations shared some similar characteristics, but there was obvious difference in ALK expression and response to EGFR and ALK inhibitors. Patient 2 with moderate ALK expression had better response to crizotinib, but inferior response to erlotinib than the patient with faint ALK expression. It was proposed [5] that ALK fusion was strongly associated with resistance to EGFR-TKIs. A patient with concomitant EGFR mutation and ALK rearrangement, demonstrated no ALK expression by IHC with an ALK rearrangement featured by an isolated 3’ FISH signal, and presented the most durable response to an EGFR-TKI [37]. While, another concurrent case of ALK rearrangement and EGFR mutation, presented ALK expression, but EGFR-TKI was not effective [36]. Until now, the most effective treatment for patients with advanced NSCLC harboring the double mutation is still an open question. However, our data provided a clue for the treatment of patients with the coexistence of EGFR mutation and ALK rearrangement by evaluating ALK expression, but further research is needed to confirm the appropriate treatment for these patients.

References
1. Lee CK, Brown C, Graff RJ, Hirsh V, Thongprasert S, et al. (2013) Impact of EGFR inhibitor in non-small cell lung cancer on progression-free and overall survival: a meta-analysis. J Natl Cancer Inst 105: 595-605.
2. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, et al. (2007) Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 448: 561-566.
3. Seto T, Kiura K, Nishio M, Nakagawa K, Maemondo M, et al. (2013) CH5424802 (RO5424802) for patients with ALK-rearranged advanced non-small-cell lung cancer (AF-061P study): a single-arm, open-label, phase 1-2 study. Lancet Oncol 14: 590-598.
4. Rodig SJ, Mino-Kenudson M, Dacic S, Yeap BY, Shaw A, et al. (2009) Unique clinicopathologic features characterize ALK-rearranged lung adenocarcinoma in the western population. Clin Cancer Res 15: 5216-5223.
5. Shigeta A, Yeap BY, Mino-Kenudson M, Dignam SR, Costa DB, et al. (2009) Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. J Clin Oncol 27: 4247-4253.
6. Camidge DR, Kono SA, Plaza A, Tan AG, Doechel RC, et al. (2010) Optimizing the detection of lung cancer patients harboring anaplastic lymphoma kinase (ALK) gene rearrangements potentially suitable for ALK inhibitor treatment. Clin Cancer Res 16: 5381-5390.
7. Zhang X, Zhang S, Yang X, Yang J, Zhou Q, et al. (2010) Fusion of EML4 and ALK is associated with development of lung adenosquamous lacking EGFR and KRAS mutations and is correlated with ALK expression. Mol Cancer 9: 188.
8. Gandhi L, Janne PA (2012) Crizotinib for ALK-rearranged non-small cell lung cancer: a new targeted therapy for a new target. Clin Cancer Res 18: 3737-3742.
9. Wallander ML, Geiersbach KB, Tripp SR, Layfield LJ (2012) Comparison of reverse transcription-polymerase chain reaction, immunohistochemistry, and fluorescence in situ hybridization methodologies for detection of echinoderm microtubule-associated proteinlike 4-anaplastic lymphoma kinase fusion-positive non-small cell lung carcinoma: implications for optimal clinical testing. Arch Pathol Lab Med 136: 796-803.
10. Tiseo M, Gehlomino F, Boggiani D, Bortesi B, Bartolotti M, et al. (2011) EGFR and EML4-ALK gene mutations in NSCLC: a case report of erlotinib-resistant patient with both concomitant mutations. Lung Cancer 71: 241-243.
11. Wang Z, Zhang X, Bai H, Zhao J, Zhao M, et al. (2012) EML4-ALK rearrangement and its clinical significance in Chinese patients with advanced non-small cell lung cancer. Oncology 83: 248-256.
12. Kuo YW, Wu SG, Ho CC, Shih JY (2010) Good response to gefitinib in lung adenocarcinoma harboring coexisting EML4-ALK fusion gene and EGFR mutation. J Thorac Oncol 5: 2039-2043.
13. Martelli MP, Sozzi G, Hernandez I, Petrossi V, Navarro A, et al. (2009) EML4-ALK rearrangement in non-small cell lung cancer: a new tumor entity? J Thorac Oncol 4: 977-981.
14. Kim H, Yoo SR, Cho YJ, Park JJ, Hu X, et al. (2011) Detection of ALK gene rearrangement in non-small cell lung cancer: a comparison of fluorescence in situ hybridization and chromogenic in situ hybridization with correlation of ALK protein expression. J Thorac Oncol 6: 1365-1366.
15. Ou SH, Barbélet CH, Mino-Kenudson M, Cui J, Iafrate AJ (2012) Crizotinib for the treatment of ALK-rearranged non-small cell lung cancer: a success story to usher in the second decade of molecular targeted therapy in oncology. Oncologist 17: 1351-1357.
16. Thunnissen E, Bubendorf L, Dietel M, Ellingerger G, Kerr K, et al. (2012) EML4-ALK testing in non-small cell carcinomas of the lung: a review with recommendations. Virchows Arch 461: 245-257.
17. Marchetti A, Arlidizzi A, Papotti M, Crino L, Rossi G, et al. (2013) Recommendations for the analysis of ALK gene rearrangements in non-small cell lung cancer: a consensus of the Italian Association of Medical Oncology and the Italian Society of Pathology and Cytopathology. J Thorac Oncol 8: 352-358.
18. Beasley MB, Brambilla E, Travis WD (2005) The 2004 World Health Organization classification of lung tumors. Semin Roentgenol 40: 90-97.
19. Travis WD, Brambilla E, Nogushi M, Nicholson AG, Geisinger KK, et al. (2011) International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma. J Thorac Oncol 6: 244-285.
20. Yi ES, Boland JM, Malezewski JJ, Roden AC, Oliveira AM, et al. (2011) Correlation of IHC and FISH for ALK gene rearrangement in non-small cell lung carcinoma: IHC score algorithm for FISH. J Thorac Oncol 6: 459-465.
21. Park HS, Lee JK, Kim DW, Kulig K, Kim TM, et al. (2012) Immunohistochemical screening for anaplastic lymphoma kinase (ALK) rearrangement in advanced non-small cell lung cancer patients. Lung Cancer 77: 288-292.
22. Wong DW, Leung EL, So KK, Tam IV, Shioe AD, et al. (2009) The EML4-ALK fusion gene is involved in various histologic types of lung cancers from smokers with wild-type EGFR and KRAS. Cancer 115: 1723-1733.
23. Takahashi T, Sonobe M, Kobayashi M, Yoshizawa A, Menju T, et al. (2010) Clinicopathologic features of non-small-cell lung cancer with EML4-ALK fusion gene. Ann Surg Oncol 17: 889-897.
24. Lee JK, Park HS, Kim DW, Kulig K, Kim TM, et al. (2012) Comparative analyses of overall survival in patients with anaplastic lymphoma kinase-positive and matched wild-type advanced non-small cell lung cancer. Cancer 118: 3579-3586.
25. Yang P, Kulig K, Boland JM, Erickson-Johnson MR, Oliveira AM, et al. (2012) Worse disease-free survival in never-smokers with ALK+ lung adenocarcinoma. J Thorac Oncol 7: 90-97.
26. Wu SG, Kuo YW, Chang YL, Shih JY, Chen YH, et al. (2012) EML4-ALK translocation predicts better outcome in lung adenocarcinoma patients with wild-type EGFR. J Thorac Oncol 7: 98-104.
27. Shaw AT, Yeap BY, Solomon BJ, Riely GJ, Gainer J, et al. (2011) Effect of crizotinib on overall survival in patients with advanced non-small-cell lung cancer harbouring ALK gene rearrangement: a retrospectiveanalysis. Lancet Oncol 12: 1004-1012.
28. Camidge DR, Kono SA, Lu X, Okayama S, Baron AE, et al. (2011) Anaplastic lymphoma kinase gene rearrangements in non-small-cell lung cancer are associated with prolonged progression-free survival on pemetrexed. J Thorac Oncol 6: 774-780.
29. Lee JO, Kim TM, Lee SH, Kim DW, Kim S, et al. (2011) Anaplastic lymphoma kinase translocation: a predictive biomarker of pemetrexed in patients with non-small cell lung cancer. J Thorac Oncol 6: 1474-1480.
30. Chibbaro D, Suzuki R (2011) More on crizotinib. N Engl J Med 364: 776-777; author reply 778.
31. Sun JM, Choi YL, Won JK, Hirsch FR, Ahn JS, et al. (2012) A dramatic improvement on pemetrexed in patients with non-small cell lung cancer harbouring ALK gene rearrangement: a retrospective analysis. J Thorac Oncol 12: 1004-1012.
32. Takahashi T, Sonobe M, Kobayashi M, Yoshizawa A, Menju T, et al. (2010) Clinicopathologic features of non-small-cell lung cancer with EML4-ALK fusion gene. Ann Surg Oncol 17: 889-897.
33. Lee JK, Park HS, Kim DW, Kulig K, Kim TM, et al. (2012) Comparative analyses of overall survival in patients with anaplastic lymphoma kinase-positive and matched wild-type advanced non-small cell lung cancer. Cancer 118: 3579-3586.
34. Yang P, Kulig K, Boland JM, Erickson-Johnson MR, Oliveira AM, et al. (2012) Worse disease-free survival in never-smokers with ALK+ lung adenocarcinoma. J Thorac Oncol 7: 90-97.
35. Wu SG, Kuo YW, Chang YL, Shih JY, Chen YH, et al. (2012) EML4-ALK translocation predicts better outcome in lung adenocarcinoma patients with wild-type EGFR. J Thorac Oncol 7: 98-104.
36. Shaw AT, Yeap BY, Solomon BJ, Riely GJ, Gainer J, et al. (2011) Effect of crizotinib on overall survival in patients with advanced non-small-cell lung cancer harbouring ALK gene rearrangement: a retrospectiveanalysis. Lancet Oncol 12: 1004-1012.
34. Ren S, Chen X, Kuang P, Zheng L, Su C, et al. (2012) Association of EGFR mutation or ALK rearrangement with expression of DNA repair and synthesis genes in never-smoker women with pulmonary adenocarcinoma. Cancer 118: 5588–5594.

35. Koivunen JP, Mermel C, Zejnullahu K, Murphy C, Lifshits E, et al. (2008) EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. Clin Cancer Res 14: 4275–4283.

36. Miyanaga A, Shimizu K, Noro R, Seike M, Kitamura K, et al. (2013) Activity of EGFR-tyrosine kinase and ALK inhibitors for EML4-ALK-rearranged non-small-cell lung cancer harbored coexisting EGFR mutation. BMC Cancer 13: 262.

37. Popat S, Vieira de Araujo A, Min T, Swansbury J, Dainton M, et al. (2011) Lung adenocarcinoma with concurrent exon 19 EGFR mutation and ALK rearrangement responding to erlotinib. J Thorac Oncol 6: 1962–1963.