Detection of EML4-ALK fusion gene and features associated with EGFR mutations in Chinese patients with non-small-cell lung cancer

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Purpose: Echinoderm microtubule-associated protein-like 4–anaplastic lymphoma kinase (EML4-ALK) and epidermal growth factor receptor (EGFR) define specific molecular subsets of lung cancer with distinct clinical features. We aimed at revealing the clinical features of EML4-ALK fusion gene and EGFR mutation in non-small-cell lung cancer (NSCLC).

Methods: We enrolled 694 Chinese patients with NSCLC for analysis. EML4-ALK fusion gene was analyzed by real-time polymerase chain reaction, and EGFR mutations were analyzed by amplified refractory mutation system.

Results: Among the 694 patients, 60 (8.65%) patients had EML4-ALK fusions. In continuity correction χ² test analysis, EML4-ALK fusion gene was correlated with sex, age, smoking status, and histology, but no significant association was observed between EML4-ALK fusion gene and clinical stage. A total of 147 (21.18%) patients had EGFR mutations. In concordance with previous reports, EGFR mutation was correlated with age, smoking status, histology, and clinical stage, whereas patient age was not significantly associated with EGFR mutation. Meanwhile, to our surprise, six (0.86%) patients had coexisting EML4-ALK fusions and EGFR mutations.

Conclusion: EML4-ALK fusion gene defines a new molecular subset in patients with NSCLC. Six patients who harbored both EML4-ALK fusion genes and EGFR mutations were identified in our study. The EGFR mutations and the EML4-ALK fusion genes are coexistent.

Keywords: NSCLC, EML4-ALK fusion gene, EGFR mutation, RT-PCR

Introduction

Lung cancer is one of the most common malignancies and is a leading cause of cancer-related deaths worldwide, and it is associated with a 5-year survival rate of less than 15%.1,2 Non-small-cell lung cancer (NSCLC) accounts for approximately 80%–85% of lung cancer. Although progress has been made in traditional surgery, chemotherapy, and radiotherapy for the treatment of advanced lung cancer, clinical outcomes are still considered unsatisfactory, and the median survival rates are still limited.3 In recent years, with the development of molecular biology and human genomics, people are paying growing attention to tumor pathogenesis; molecular targeted therapy with high specificity and little adverse reactions has become an important therapeutic modality for lung cancer;4 and people have achieved great success, especially in treating patients with NSCLC. A successful example is the identification of the epidermal growth factor receptor (EGFR) mutation as a reliable, predictive biomarker for EGFR tyrosine kinase inhibitors (TKIs) treatment. In patients carrying the EGFR mutation who have been previously untreated, EGFR-TKIs have been demonstrated to be superior to cytotoxic chemotherapy.5
A fusion protein between the *echinoderm microtubule-associated protein-like 4 (EML4)* and the *anaplastic lymphoma kinase (ALK)* in NSCLC was first identified by Soda in 2007. EML4-ALK is a fusion gene, which represents a new molecular target. It has been reported that the incidence of ALK rearrangement ranged from approximately 3% to 13% in unselected or selected patients with NSCLC. The EML4-ALK translocation can result in constitutive ALK kinase activity and represents an oncogenic addiction pathway in lung cancer. EML4-ALK possesses potent oncogenic activity both in vitro and in vivo, and the tumor can quickly fade after administration of ALK-TKIs. Crizotinib, a small-molecule ALK-TKI, showed significant benefit to patients with advanced NSCLC with EML4-ALK fusion in clinical trials and has been approved by the US Food and Drug Administration for these patients.

The present study focuses on the relationship between EML4-ALK fusion and EGFR mutation. Previous studies proved that EML4-ALK fusion gene and EGFR mutation have largely been reported to be mutually exclusive. During the detection of EML4-ALK, patients with coexisting EML4-ALK fusion gene and EGFR mutation have been reported in some clinical cases. Therefore, we should pay close attention to the coexisting type rates that occurred in our study. In this study, we analyzed the relationship between EML4-ALK fusion gene and EGFR mutation. Meanwhile, we investigated the prevalence of two major mutations and their clinical characteristics in 694 unselected Chinese patients with NSCLC. These results can provide theoretical basis and important reference for individualized treatment in NSCLC.

**Materials and methods**

**Patients**

Fresh tumor specimens were obtained from 694 consecutive Chinese patients with NSCLC who underwent surgery at the Department of Thoracic Surgery, Tangdu Hospital (Xi’an, Shaanxi, People’s Republic of China) from March 2012 to September 2014. The study was approved by the Review Board of the Fourth Military Medical University. Written informed consent was obtained from each patient prior to testing.

**Clinical characteristics**

Medical records of all patients including age, sex, smoking status, histology, and clinical stage were acquired. All patients suffered from NSCLC for the first time, and none of them had received any neoadjuvant chemotherapy or radiotherapy before surgery. Tumor histology was classified according to the 3rd World Health Organization (WHO)/International Association for the Study of Lung Cancer (IASLC) criteria. Tumor stages were determined using Version 7 of the IASCL (IASCL, Aurora, CO, USA). The histological subtypes of all patients were reassessed by at least two lung pathologists.

**EGFR mutation and EML4-ALK fusion gene analysis**

EGFR mutation detection: Genomic DNA was isolated and purified from fresh tumor specimens using TIANamp Genomic DNA Kit (Taigen Biotech, Beijing, People’s Republic of China) according to the manufacturer’s instructions. After that, EGFR mutations were analyzed using the principle of amplified refractory mutation system (ARMS), following the protocol of the AmoyDx EGFR Gene Mutation Detection Kit (Amoy Diagnostics, Haicang, Xiamen, People’s Republic of China) and covering 29 EGFR mutation hotspots from exons 18 to 21. The assay was carried out according to the manufacturer’s instructions using the MX3005P (Stratagene, La Jolla, CA, USA) real-time polymerase chain reaction (RT-PCR) system. PCR was performed with initial denaturation at 95°C for 5 minutes, followed by 15 cycles of amplification (at 95°C for 25 s, 64°C for 20 s, and 72°C for 20 s) and a final denaturation followed by 31 cycles of amplification (at 93°C for 25 s, 60°C for 35 s, and 72°C for 20 s), and the FAM and HEX signals were collected at 60°C. The results were analyzed according to the criteria defined by the manufacturer’s instructions. Positive results were defined as Ct (sample) – Ct (control) < Ct (cut-off).

EML4-ALK fusion gene detection: Total RNAs were extracted from fresh cancer tissues using an E.Z.N.A Total RNA Kit I (OMEGA Bio-tek, Norcross, GA, USA) following the manufacturer’s instructions. EML4-ALK fusion genes were detected by RT-PCR, following the protocol given in the AmoyDx EML4-ALK Fusion Gene Detection Kit (Amoy Diagnostics). The assay was carried out according to the manufacturer’s instructions with the MX3005P (Stratagene) RT-PCR system. The PCR conditions consisted of initial denaturation at 95°C for 5 minutes, followed by 15 cycles of amplification (at 95°C for 25 s, 64°C for 20 s, and 72°C for 20 s) and final denaturation followed by 31 cycles of amplification (at 93°C for 25 s, 60°C for 35 s, and 72°C for 20 s), and the FAM signal was collected at 60°C. The results were analyzed according to the criteria defined by the manufacturer’s instructions. Positive results were defined as Ct (sample) < 30.

**Statistical analyses**

Statistical analysis was performed using SPSS Version 16.0 Statistical Software (SPSS Inc., Chicago, IL, USA). The
\( \chi^2 \) test or Fisher’s exact test was used to assess the relationship between the presence of \( EGFR \) mutation and clinical features as well as \( EML4-ALK \) fusion gene and the clinical features. All \( P \)-values were based on a two-sided hypothesis, and the statistical significance was set at \( P<0.05 \) for all analyses.

**Results**

**Correlation between \( EML4-ALK \) fusion gene and clinicopathologic characteristics**
The clinical characteristics of these 694 patients are described in Table 1. \( EML4-ALK \) fusion genes were identified in 60 (8.65%) of all cases. \( EML4-ALK \) fusion gene was correlated with age, sex, smoking status, and histology, but no significant association was observed between \( EML4-ALK \) fusion gene and clinical stage.

**The proportion of clinicopathologic characteristics in patients with \( EML4-ALK \) fusion genes**
Among the 60 patients with \( EML4-ALK \) fusion gene, 41 (68.33%) were of lower median age and 19 (31.67%) were of higher median age; 32 (53.33%) were male and 28 (46.67%) were female; 34 (56.67%) were never smokers and 26 (43.33%) were smokers; 14 (23.34%) patients were in stage I, eight (13.33%) were in stage II, 26 (43.34%) were in stage III, and 12 (20%) were in stage IV; according to the IASLC, 42 (70%) were adenocarcinomas, eight (13.33%) were squamous cell carcinomas, seven (11.67%) were adenosquamous carcinomas, and three (5%) were other specified carcinomas.

**Correlation between \( EGFR \) mutation and clinicopathologic characteristics**
As shown in Table 2, of the 694 patients, we identified 147 (21.18%) patients who harbored \( EGFR \) mutations. Clinical and pathological characteristics were analyzed for association with \( EGFR \) mutation. Sex, smoking status, histology, and clinical stage were associated with \( EGFR \) mutation, but no significant association was found between \( EGFR \) mutation and age.

**The proportion of clinicopathologic characteristics in patients with \( EGFR \) mutations**
Of the 147 patients with \( EGFR \) mutation, 77 (52.38%) were of lower median age and 70 (47.62%) were of higher median age; 64 (43.54%) were male and 83 (56.46%) were

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**Table 1** Association of \( EML4-ALK \) fusion gene with clinicopathological characteristics

| Characteristics | Totala | \( EML4-ALK \) fusiona | \( P \)-value |
|-----------------|--------|------------------------|--------------|
| Total patients  | 694    | 60                     |              |
| Sex             |        |                        | 0.000        |
| Male            | 504    | 32 (6.35)              |              |
| Female          | 190    | 28 (14.74)             |              |
| Age (years)     |        |                        | 0.002        |
| <60             | 340    | 41 (12.06)             |              |
| ≥60             | 354    | 19 (5.37)              |              |
| Smoking history |        |                        | 0.001        |
| Never           | 259    | 34 (13.13)             |              |
| Everb           | 435    | 26 (5.98)              |              |
| Histology       |        |                        | 0.000        |
| ADC             | 315    | 42 (13.33)             |              |
| SCC             | 255    | 8 (3.14)               |              |
| ADSQ            | 68     | 7 (10.29)              |              |
| Other NSCLCc    | 56     | 3 (5.36)               |              |
| Clinical stage  |        |                        | 0.063        |
| I               | 208    | 14 (6.73)              |              |
| II              | 152    | 8 (5.26)               |              |
| III             | 248    | 26 (10.48)             |              |
| IV              | 86     | 12 (13.95)             |              |

**Notes:** aValues in parentheses indicate percentage. bA person who smoked more than 100 cigarettes in his/her past history was defined as an ever smoker. cOther types included sarcomatoid, large cell, and mucocleidemoid.

**Abbreviations:** \( EML4-ALK \), echinoderm micrutilbule-associated protein-\( \alpha \)-4–anaplastic lymphoma kinase; ADC, adenocarcinoma; SCC, squamous cell carcinoma; ADSQ, adenosquamous carcinoma; NSCLC, non-small-cell lung cancer.

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**Table 2** Association of \( EGFR \) mutation with clinicopathological characteristics

| Characteristics | Totala | \( EGFR \) mutationa | \( P \)-value |
|-----------------|--------|----------------------|--------------|
| Total patients  | 694    | 147 (21.18)          |              |
| Sex             |        |                      | 0.000        |
| Male            | 504    | 64 (12.70)           |              |
| Female          | 190    | 83 (43.68)           |              |
| Age (years)     |        |                      | 0.355        |
| <60             | 340    | 77 (22.65)           |              |
| ≥60             | 354    | 70 (19.77)           |              |
| Smoking history |        |                      | 0.000        |
| Never           | 259    | 105 (40.54)          |              |
| Everb           | 435    | 39 (8.97)            |              |
| Histology       |        |                      | 0.000        |
| ADC             | 315    | 114 (36.19)          |              |
| SCC             | 255    | 11 (4.31)            |              |
| ADSQ            | 68     | 17 (25)              |              |
| Other NSCLCc    | 56     | 5 (8.93)             |              |
| Clinical stage  |        |                      | 0.008        |
| I               | 208    | 52 (25)              |              |
| II              | 152    | 17 (11.18)           |              |
| III             | 248    | 57 (22.98)           |              |
| IV              | 86     | 21 (24.42)           |              |

**Notes:** aValues in parentheses indicate percentage. bA person who smoked more than 100 cigarettes in his/her past history was defined as an ever smoker. cOther types included sarcomatoid, large cell, and mucocleidemoid.

**Abbreviations:** \( EGFR \), epidermal growth factor receptor; ADC, adenocarcinoma; SCC, squamous cell carcinoma; ADSQ, adenosquamous carcinoma; NSCLC, non-small-cell lung cancer.
female; 105 (71.43%) were never smokers and 39 (28.57%) were smokers; 52 (35.37%) patients were in stage I, 17 (11.56%) were in stage II, 57 (38.78%) were in stage III, and 21 (14.29%) were in stage IV; according to the IASLC, 114 (77.55%) were adenocarcinomas, eleven (7.48%) were squamous cell carcinomas, 17 (11.56%) were adenosquamous carcinomas, and five (3.41%) were other specified carcinomas; in addition, three cases exhibited double mutations in EGFR exons, including E(G719X; L861Q), E(G719X; S768I), and E(19-del; T790M).

**Characteristics in patients with NSCLC with EGFR/EML4-ALK coalterations**

Six patients (0.86%) had coexistence of EGFR mutation and EML4-ALK fusion gene. Among the patients with coalterations, three (50%) had adenocarcinomas, one (16.7%) had mucoepidermoid carcinoma, and two (33.3%) had sarcomatoid carcinomas; four (66.7%) were male and two (33.3%) were female; four (66.7%) had a lower median age and two (33.3%) had a higher median age; all the six (100%) patients were never smokers; two (33.3%) patients were in stage I, one (16.7%) was in stage II, two (33.3%) were in stage III, and one (16.7%) was in stage IV. The clinical characteristics of the patients with coalterations are shown in Table 3. Typical results of patients with coalterations are shown in Figure 1.

**Discussion**

With the development of molecular medicine, the application of several molecular targeting drugs in lung cancer has garnered increased attention, and remarkable successes have been reported in several patients with NSCLC. Targeted biological therapies have led patients with advanced NSCLC to new therapeutic options. At present, EGFR-TKIs, such as gefitinib and erlotinib, have shown great efficacy in patients with NSCLC who have activation mutations in the EGFR gene.20,21 Recently, the discovery of the EML4-ALK fusion gene, which was first reported in NSCLC in 2007, represents a new molecular target. EML4-ALK fusion has been reported to suppress the growth of EML4-ALK-fusion-gene-positive cells.15,22 Thus, the ALK inhibitors can be effective for patients with NSCLC whose tumors contain an EML4-ALK fusion gene. There are three usual methods for genetic detection – fluorescence in situ hybridization (FISH), RT-PCR, and immunohistochemistry.23–25 We choose different methods according to different specimen types. RT-PCR is a highly sensitive and specific technique, and since fresh tumor samples after lung resection could be used in our study; we adopted RT-PCR as a detection method.

Previous studies reported that the incidence of EML4-ALK fusion gene varied from 3% to 5% in unselected populations, but the frequency is up to 13.5% in selected populations.16,26 The incidence of EML4-ALK fusion gene in Chinese patients with NSCLC was approximately 2.6%–11.9%, but the studies had some limitations, including smaller sample size. In our study, EML4-ALK fusion gene in unselected patients was detected by RT-PCR, and the incidence of EML4-ALK fusion gene was 8.65% (60/694). The clinical characteristics of ALK-positive or -negative patients were analyzed by χ² testing, which showed significant differences in sex, age, smoking status, and histology. The proportion of EML4-ALK-positive patients seemed to be of younger age, light or never smokers, and had histology of adenocarcinomas. There are greater differences between the sexes in the patients who harbored EML4-ALK fusion gene. Shaw et al9 found that EML4-ALK fusion gene occurred more frequently in men, those of relatively young age, nonsmokers, and those with adenocarcinomas.9 However, in our study, the incidence of EML4-ALK fusion gene was significantly higher in women than in men. We suggest that the patients with NSCLC, both men and women, should be tested for EML4-ALK fusion gene.

EGFR mutation rate has obvious regional differences in NSCLC; it is reported that the EGFR-positive rate was found to be 30% in Asian patients27 but only 15% in American patients,28 and EGFR mutation was detected predominantly in Asian people, females, light or never smokers, and adenocarcinomas, which was consistent with previous reports.5,29 At present, the relationship between EGFR mutation and age is scarce; there is no clear explanation, and the mechanism still needs to be

**Table 3 The clinical characteristics of six patients with EML4-ALK fusions and EGFR mutations**

| Patient ID | EGFR mutation | ALK fusion | Sex | Age (years) | Smoking status | Tumor histology | Stage |
|------------|---------------|------------|-----|-------------|----------------|----------------|-------|
| 1          | E(19-del)     | Positive   | Male| 46          | Never          | Adenocarcinoma | IIA   |
| 2          | E(L858R)      | Positive   | Male| 24          | Never          | Mucoepidermoid carcinoma | IB |
| 3          | E(19-del)     | Positive   | Female| 64  | Never        | Adenocarcinoma | IB   |
| 4          | E(L861Q)      | Positive   | Male| 67          | Never          | Sarcomatoid carcinoma | IV |
| 5          | E(G719X)      | Positive   | Female| 47  | Never        | Adenocarcinoma | IIIB |
| 6          | E(19-del)     | Positive   | Male| 38          | Never          | Sarcomatoid carcinoma | IIIA |

Abbreviations: EML4-ALK, echinoderm microtubule-associated protein-like 4–anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor.
studied further. In this study, there was no statistical significance between EGFR mutation and age.

EGFR mutation and EML4-ALK fusion have some similar clinical features, for example, these patients are light or never smokers and had histology of adenocarcinomas. Although previous reports have indicated that EML4-ALK fusion gene and EGFR mutation were considered to be mutually exclusive in general, several studies have reported the coexistence of the EGFR mutation and EML4-ALK fusion gene, and the clinical features including Asian ethnicity, female, never or light smoking, adenocarcinomas, young patients with advanced cancer, and harboring EGFR mutations in

Figure 1  Representative results of patients with coalterations.
Notes: Amplification plots demonstrate the typical results of EGFR mutation and EML4-ALK fusion of patient 1 (A and B), patient 2 (C and D), and patient 3 (E and F). A, C, and E demonstrate the results of exon 19 deletion mutation, exon 21 L858R point mutation, and exon 18 G719X point mutation, respectively. B, D, and F demonstrate the results of EML4-ALK fusion, respectively.
Abbreviations: EGFR, epidermal growth factor receptor; EML4-ALK, echinoderm microtubule-associated protein-like 4–anaplastic lymphoma kinase.
In the present study, six patients had coexisting EML4-ALK fusion genes and EGFR mutations; further analysis revealed that of these six patients, four were male, three had adenocarcinomas, four were in relatively early stage of disease, four were of younger age, and EGFR mutations were either exon 19 deletion mutations (3/6), or exon 21 point mutations (2/6), or exon 18 point mutation (1/6). In general, patients need to be tested for the presence of EML4-ALK fusion gene when the EGFR mutation is negative. Given the existence of double mutation, we can detect both EML4-ALK fusion and EGFR mutation to prevent missing EML4-ALK fusion gene, and hence provide more choices to patients. EGFR-TKIs have become an indispensable and important modality for treating advanced NSCLC with an EGFR mutation. A series of driver genes of lung cancer have been identified, and EML4-ALK fusion gene has become one of the research hotspots in recent years. However, because only few cases of coexistence of EGFR mutation and EML4-ALK fusion gene have been reported to date, and further research needs to be done. It still remains unclear as to how to introduce TKIs to patients who have coexisting EML4-ALK fusion gene and EGFR mutation. The appropriate treatment for coalterative patients should be further identified in the clinical trials.

Conclusion
We investigated the frequency of EML4-ALK fusion gene and its association with clinicopathologic factors in 694 patients with NSCLC with identified EGFR mutation. Meanwhile, we found six patients had coexisting EML4-ALK fusion genes and EGFR mutations. The role of EML4-ALK fusion gene as an oncogenic driver may be more important in NSCLC than what we expected. Furthermore, our study also revealed a strong association between EML4-ALK fusion gene and sex, age, smoking status, and histology. Our data also suggested EML4-ALK status should be investigated in unexplained cases of TKI-resistance of EGFR-mutated NSCLC. To better use targeted drugs, further study focusing on specific subgroups with EML4-ALK/EGFR coalterations should be performed in future.

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Disclosure
The authors report no conflicts of interest in this work.

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