Correlation between EML4-ALK, EGFR and clinicopathological features based on IASLC/ATS/ERS classification of lung adenocarcinoma

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Abstract
To investigate the correlation between echinoderm microtubule associated protein-like 4 (EML4)-anaplastic lymphomakinase (ALK), epidermal growth factor receptor (EGFR) and clinicopathological features in patients diagnosed with lung adenocarcinoma according to International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) international multidisciplinary classification of lung adenocarcinoma.

Ninety patients diagnosed with lung adenocarcinoma underwent surgical pathological classification. Ventana immunohistochemical staining of the EML4-ALK was performed. The mutation of EGFR and EML4-ALK was detected by real-time polymerase chain reaction (RT-PCR) using the amplification refractory mutation system.

The positive rate of EML4-ALK mutation was calculated as 6.7% (6/90), dominantly occurring in patients aged < 60 years. However, it was not correlated with the gender, smoking history, maximal tumor diameter, pleural invasion, lymphatic metastasis, or clinical staging. EML-ALK fusion gene mutation was mainly associated with the predominant subtypes of acinar and solid tumors with mucin secretion. The mutation rate of EGFR was 60% (27/45). EGFR gene mutation mainly occurred in the female, those with no smoking history and tumor size < 3 cm, whereas it had no association with age, pleural invasion, lymphatic metastasis, or clinical staging. It was histologically characterized with micro papillary, lepidic, and papillary subtypes.

The mutation rate of EML4-ALK is relatively high in lung adenocarcinoma patients aged < 60 years, pathologically characterized with acinar and solid subtypes with mucin secretion. Female patients with no smoking habit, tumor size < 3 cm, pathologically characterized with micropapillary, lepidic, and papillary subtypes had a high mutation rate of EGFR.

Abbreviations: ALK = anaplastic lymphomakinase, ARMS = amplification refractory mutation system, ATS = American Thoracic Society, CT = computed tomographic, EGFR = epidermal growth factor receptor, EML4 = echinoderm microtubule associated protein like 4, ERS = European Respiratory Society, IASLC = International Association for the Study of Lung Cancer, RT-PCR = real-time polymerase chain reaction, TK = tyrosine kinase.

Keywords: correlation analysis, EGFR, EML4-ALK, gene mutation, lung adenocarcinoma

1. Introduction
Epidermal growth factor receptor (EGFR) is a receptor protein, which consists of 486 amino acids and 170 kDa in size. It possesses a single trans-membrane sequence among the extracellular and intracellular domains.[1] Recent studies have indicated that patients diagnosis with lung adenocarcinoma and EGFR mutation can obtain clinical benefits from the target therapy of gefitinib. Lung adenocarcinoma can harbor EGFR mutation, which is a protein on cell surface with intracellular tyrosine kinase (TK) activity. Due to targetable activating mutations, tumors are susceptible to erlotinib and gefitinib.[2] It has been reported that 3% to 5% of lung adenocarcinoma patients carry the translocations of the anaplastic lymphoma kinase (ALK) gene. The most common translocation leads to an aberrant fusion between the microtubule-associated protein-like 4 gene (EML4) and ALK. This event contributes to the formation of cytoplasmic chimeric protein, which provokes the oncogenic signaling pathway and serves as a therapeutic target. Compared with the second-line single agent chemotherapy, crizotinib chemotherapy can prolong the progression-free survival and enhance the objective response rate.[3]

In the year of 2011, American Thoracic Society, International Association for the Study of Lung Cancer, and European Respiratory Society jointly proposed a novel classification of lung adenocarcinoma which included multiple modifications to the previous classification criterion.[4] This classification now considers sample resection, small biopsy, and cytological specimens. Bronchial alveolar cancer and mixed-subtype adenocarcinoma are not utilized. Invasive adenocarcinomas can be classified into the acinar, lepidic, solid, papillary, and micro-papillary patterns, which offer instructions for cytological specimens and small biopsy. In this investigation, the correlation between the mutation of EML4-ALK and EGFR, and the

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clinicopathological features was evaluated in 90 patients diagnosed with lung adenocarcinoma.

2. Materials and methods

2.1. Specimen collection

The excisional samples of 90 lung adenocarcinoma patients admitted to the First Affiliated Hospital of Xi’an Jiaotong University between September 2011 and December 2014 were retrospectively analyzed. All specimens were fixed in 4% neutral formalin solution, paraffin embedding and prepared for subsequent immunohistochemical staining. Informed consents were obtained from all participants in this investigation. The study procedures comply with the ethics committee of Xi’an Jiaotong University.

2.2. Clinicopathological features

Clinicopathological parameters including age, gender, smoking history, clinical staging, histological subtype, tumor size, pleural invasion, and lymphatic metastasis were recorded. The HE staining sections were examined and reviewed for histological subtyping strictly according to IASLC/ATS/ERS international multidisciplinary classification of lung adenocarcinoma, as illustrated in Figure 1.

![Figure 1](image_url)

**Figure 1.** Histological type of infiltrative lung adenocarcinoma (HE ×20). (A) Leptidic subtype; (B) acinar subtype; (C) papillary subtype; (D) micropapillary subtype; (E) solid subtype complicated with mucin secretion; (F) infiltrative mucus adenocarcinoma.
2.3. Detection of EML4-ALK fusion gene mutation

The findings of Ventana immunohistochemical staining were re-evaluated according to the revised diagnostic criteria of nonsmall cell lung cancer with positive ALK proposed by Chinese Society of Clinical Oncology in 2013. ALK mutation was defined as the strong staining of the granule cellular cytoplasm in the tumor cells, no staining was considered as wild type.

2.4. DNA extraction and EGFR gene mutation

The 4-μm thick sections were placed into 1.5-mL centrifuge tube, dewaxing using xylene and supplemented with 1-mL absolute ethyl alcohol, gently mixed and maintained at room temperature for 5 minutes and subsequently centrifuged at 14000 r/min for 3 minutes. The supernatant was discarded. Absolute ethyl alcohol was added to eliminate the xylene. The tube was open and maintained at room temperature to thoroughly volatilize the ethanol. DNA extraction was performed strictly according to the manufacturers’ instructions (Qiagen DNA mini kit, serial No. 51304). The concentration and purity of DNA were assessed at the wavelength of OD260 and OD280. The mutation of EGFR was detected by using the amplification refractory mutation system (ARMS) from Amoy Diagnostics Co., Ltd. (Xiamen, China). Quantitative PCR amplification was performed using BIO-RAD CFX96 PCR system.

2.5. Statistical analysis

All statistical analysis was performed using SPSS 20.0 statistical software (SPSS Inc., Chicago) and SAS software (Statistical analysis system, SAS Institute Inc.). Enumeration data were statistically compared using chi-square test. When $1 < T < 5$, continuously-corrected chi-square test was conducted. Paired enumeration data were statistically compared using McNemar chi-square test. When $T < 1$, Fisher exact test was performed using the SAS statistical software. A $P$ value of $< .05$ was considered as statistical significance.

3. Results

3.1. Baseline data

Ninety patients were diagnosed with infiltrative or mutated adenocarcinoma including 8 (8.9%) with lepidic lung adenocarcinoma, 51 (56.7%) with acinar lung adenocarcinoma, 11 (12.2%) with papillary lung adenocarcinoma, 3 (3.3%) with micropapillary lung adenocarcinoma, 11 (12.2%) with solid subtype complicated with mucin secretion and 6 (6.7%) with infiltrative mucus adenocarcinoma, as illustrated in Figure 1.

3.2. Ventana immunohistochemical staining of EML4-ALK

The positive rate of EML4-ALK fusion gene mutation was calculated as 6.7% (6/90), as revealed in Figure 2. Six (14.6%) among 41 patients aged $< 60$ years had EML4-ALK fusion gene mutation, significantly higher than 0.0% in 49 cases aged $\geq 60$ years who had no EML4-ALK fusion gene mutation ($P= .019$), prompting that the mutation rate of EML4-ALK fusion gene in patients aged $< 60$ years was relatively high. No correlation was documented between EML4-ALK gene mutation and gender, smoking history, maximal tumor size, pleural invasion, lymphatic metastasis or clinical staging, as illustrated in Table 1.

3.3. Correlation between EML4-ALK fusion gene and histological subtype

Among 51 patients with acinar histological subtype, 5 (9.8%) had EML4-ALK fusion gene mutation. One among 11 cases (9.1%) of solid subtype complicated with mucin secretion had EML4-ALK fusion gene mutation. No EML4-ALK fusion gene mutation was noted in alternative histological subtypes. The positive rate of EML4-ALK fusion gene mutation significantly differed among different histological subtypes ($P= .042$), as illustrated in Table 2.

3.4. EGFR gene mutation

Among 90 patients diagnosed with lung adenocarcinoma, 45 received EGFR mutation detection and 27 were detected to have EGFR mutation with a positive rate of EGFR mutation of 60%, as illustrated in Figure 3.

3.5. Correlation between EGFR gene mutation and clinicopathological parameters

The positive rate of EGFR gene mutation in male patients was calculated as 38.9% (7/18), significantly lower compared with 74.1% (20/27) in their female counterparts ($P=.018$).

Figure 2. Ventana immunohistochemical staining of ALK (×200). (A) Solid adenocarcinoma with mucin secretion; (B) acinar adenocarcinoma.
positive rate of EGFR gene mutation in smokers was calculated as 37.5% (6/16), significantly lower compared with 72.4% (21/29) in the nonsmokers ($P = .022$). The median tumor size was selected for grouping because the maximal tumor size was abnormally distributed. In the patients with tumor size < 3 cm, the positive rate of EGFR gene mutation was 78.9% (15/19), considerably higher compared with 46.2% (12/26) in their counterparts with tumor size ≥ 3 cm ($P = .027$). As illustrated in Table 1, EGFR gene

**Table 1**

Correlation among EML4-ALK gene fusion status, EGFR gene mutation, and clinical parameters.

| Clinical parameters | EML4-ALK gene | EGFR gene | EML4-ALK gene | EGFR gene |
|---------------------|---------------|-----------|---------------|-----------|
|                     | Number of mutation cases | Total number of cases | Positive rate (%) | $P$ value | Number of mutation cases | Total number of cases | Positive rate (%) | $P$ value |
| Age                 |               |           |               |           |               |           |               |           |
| < 60 years          | 6             | 41        | 14.6          | .019      | 12            | 22        | 54.5          | .465      |
| ≥ 60 years          | 0             | 49        | 0.0           | 15        | 23            | 65.2      |               |           |
| Gender              |               |           |               |           |               |           |               |           |
| Male                | 2             | 37        | 5.4           | 1.000     | 7             | 18        | 38.9          | .018      |
| Female              | 4             | 53        | 7.5           |           | 20            | 27        | 74.1          |           |
| Smoking history     |               |           |               |           |               |           |               |           |
| Yes                 | 1             | 32        | 3.1           | .576      | 6             | 16        | 37.5          | .022      |
| No                  | 5             | 58        | 8.6           |           | 21            | 29        | 72.4          |           |
| Maximal tumor size  |               |           |               |           |               |           |               |           |
| < 3 cm              | 3             | 37        | 8.1           | .977      | 15            | 19        | 78.9          | .027      |
| ≥ 3 cm              | 3             | 53        | 5.7           |           | 12            | 26        | 46.2          |           |
| Pleural invasion    |               |           |               |           |               |           |               |           |
| Yes                 | 6             | 63        | 9.5           | .231      | 19            | 33        | 57.6          | .836      |
| No                  | 0             | 27        | 0.0           |           | 8             | 12        | 66.7          |           |
| Lymphatic metastasis|               |           |               |           |               |           |               |           |
| Yes                 | 2             | 23        | 8.7           | 1.000     | 4             | 9         | 44.4          | .494      |
| No                  | 4             | 67        | 6.0           |           | 23            | 36        | 63.9          |           |
| Clinical staging    |               |           |               |           |               |           |               |           |
| I/II                | 3             | 62        | 4.8           | .563      | 20            | 23        | 87.0          | 1.000     |
| III/IV              | 3             | 28        | 10.7          |           | 7             | 12        | 58.3          |           |

**Table 2**

Correlation among EML4-ALK gene fusion status, EGFR gene mutation, and histological subtype.

| IASLC/ATS/ERS international multidisciplinary classification | EML4-ALK gene | EGFR gene | EML4-ALK gene | EGFR gene |
|-------------------------------------------------------------|---------------|-----------|---------------|-----------|
|                                                             | Number of mutation cases | Total number of cases | Positive rate (%) | $P$ value | Number of mutation cases | Total number of cases | Positive rate (%) | $P$ value |
| Dominantly lepidic subtype                                  | 0             | 8         | 0.0           | .042      | 5             | 6         | 83.3          | .000      |
| Dominantly acinar subtype                                  | 5             | 51        | 9.8           |           | 13            | 22        | 59.1          |           |
| Dominantly papillary subtype                               | 0             | 11        | 0.0           |           | 6             | 8         | 75.0          |           |
| Dominantly micropapillary subtype                           | 0             | 3         | 0.0           |           | 2             | 2         | 100.0         |           |
| Dominantly solid subtype complicated with mucin secretion  | 1             | 11        | 9.1           |           | 1             | 4         | 25.0          |           |
| Infiltrative mucus adenocarcinoma                           | 0             | 6         | 0.0           |           | 0             | 3         | 0.0           |           |

Figure 3. Signaling graph of EGFR gene mutation. (A) Internal control signal; (B) external and mutation signals.
mutation was not associated with age, gender, pleural invasion, lymphatic metastasis, or clinical staging.

3.6. Correlation between EGFR gene mutation and histological subtype

Among 90 patients with lung adenocarcinoma, 45 cases received EGFR gene detection. In this subgroup, the positive rate of EGFR gene mutation in patients with predominant patterns of micropapillary, lepidic, and papillary subtypes was relatively higher compared with that in other types ($P = .000$), as illustrated Table 2.

4. Discussion

In recent years, significant progression has been obtained in the molecular biological research of lung adenocarcinoma, especially for lung adenocarcinoma patients complicated with EGFR, KRAS, and EMIL-ALK mutation. It has been demonstrated that lung adenocarcinoma patients complicated with EGFR and EML-ALK are susceptible to the target therapy of gefitinib, significantly enhancing the clinical prognosis. Previous studies have found that the specific histological components are associated with certain molecular changes.

In this investigation, EML-ALK gene mutation was mainly observed in patients aged < 60 years, which is consistent with previous findings.[16] Shaw et al.[7] reported that the positive rate of EML-ALK fusion gene mutation in male patients was 22.9%, significantly higher compared with 8.6% in female counterparts. However, no correlation was documented between EML-ALK gene mutation and gender in our study. This discrepancy probably results from that the fact that sampling screening or relatively low positive rate of EML-ALK fusion gene in the study of Shaw. In addition, the sampling size in present investigation is insufficient, which may lead to data deviation. The association between EML-ALK fusion gene and smoking history remains elusive. Previous studies of Western population have demonstrated that the positive rate of EML-ALK fusion gene mutation in smokers is significantly lower compared with that in nonsmokers.[7,8] However, in this study, we found no correlation between EML-ALK fusion gene and smoking history in Chinese. We hypothesized that the difference in the association between EML-ALK fusion gene and smoking history probably results from the population disparity. Moreover, no apparent association was noted among EML-ALK fusion gene mutation, maximal tumor size, lymphatic metastasis, and tumor staging, which is consistent with previous investigations.[7,9,10]

Prior to the introduction of IASLC/ATS/ERS international multidisciplinary classification of lung adenocarcinoma, WHO classification criterion is mainly adopted to evaluate the relationship between histological type of lung adenocarcinoma and EML-ALK fusion gene. In Western population, EML-ALK fusion gene mutation is mainly detected in patients diagnosed with solid adenocarcinoma and signet-ring cell lung carcinoma. Although histological subtype does not significantly differ, the positive rate of EML-ALK fusion gene mutation in patients with solid adenocarcinoma complicated with signet-ring cell carcinoma is significantly higher compared with those in alternative histological subtypes.[11] In Asian population, Jokoji et al.[12] and Sakairi et al.[10] have demonstrated that EML-ALK fusion gene mutation is dominantly detected in patients with mucus adenocarcinoma and cribriform adenocarcinoma complicated with a high quantity of extracellular mucus. Nevertheless, after the release of IASLC/ATS/ERS international multidisciplinary classification of lung adenocarcinoma, EML-ALK fusion gene mutation is mainly detected in patients with micropapillary, acinar and infiltrative mucus adenocarcinoma. In current investigation, EML-ALK fusion gene mutation is dominantly detected in patients with acinar and solid adenocarcinoma complicated with mucus, which is consistent with previous findings.[13]

In this study, EGFR gene mutation is preferentially detected in the female patients, nonsmokers and those with maximal tumor size < 3 cm, whereas it is not correlated with age, pleural invasion, lymphatic metastasis or tumor staging, which is almost consistent with previous findings.[14] After the release of IASLC/ATS/ERS international multidisciplinary classification of lung adenocarcinoma, previous investigations have demonstrated that EGFR gene mutation is mainly detected in patients with lepidic, micropapillary, and acinar subtypes, whereas the mutation rate is extremely low in patients with solid adenocarcinoma, which is almost consistent with the outcomes of current study.[15] Yoshizawa et al.[16] reported that EGFR gene mutation is commonly detected in patients diagnosed with in situ adenocarcinoma and infiltrative micro-adenocarcinoma. These findings imply that IASLC/ATS/ERS international multidisciplinary classification of lung adenocarcinoma can reflect the gene typing of lung adenocarcinoma. Yang et al.[17] quantitatively investigated the CT scan characteristics, which are correlated with the mutations of 3 driver genes in patients diagnosed with lung adenocarcinomas patients in a Chinese cohort and have demonstrated that it is important to understand ground-glass opacity lesions in patients with lung adenocarcinomas and explore relevant molecular biomarkers, which can add new evidence to select the optimal therapy for lung adenocarcinoma with ground-glass opacity lesions revealed by CT scan. Nakamura et al.[18] have analyzed the relationships between the subtypes and EGFR, and have found that the outcomes after the lung adenocarcinoma was fully removed can be predicted according to the subtype classification. Since EGFR mutations can be detected in all subtypes, it is essential to perform the mutation analysis for selecting the recurrent patients who can be effectively treated with EGFR-tyrosine kinase inhibitor.

Nevertheless, the findings of current investigation remain to be validated by subsequent research with larger sample size. Besides, the survival data of the lung adenocarcinoma patients should be further confirmed by more investigations.

5. Conclusion

To conclude, the mutation rate of EML-ALK fusion gene is relatively high in patients aged < 60 years, acinar and solid subtypes complicated with mucin secretion. Meantime, the positive rate of EGFR gene mutation is higher in female patients, nonsmokers, those with maximal tumor size < 3 cm, micropapillary, lepidic, papillary, and acinar subtypes of lung adenocarcinoma compared with their counterparts.

Author contributions

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