**EML4-ALK** translocation is associated with early onset of disease and other clinicopathological features in Chinese female never-smokers with non-small-cell lung cancer

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**Abstract.** Non-small-cell lung cancer (NSCLC) with echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (**EML4-ALK**) translocation is resistant to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), including gefitinib and erlotinib, but responds to the ALK-TKI crizotinib. Characterization of **EML4-ALK** translocation may provide invaluable information to facilitate disease diagnosis and improve the outcome of customized treatment. Although the occurrence of **EML4-ALK** translocation is likely to be affected by the smoking habits and gender of patients, the translocation has not been characterized extensively in female never-smokers with NSCLC. Therefore, 280 female never-smokers that were diagnosed with NSCLC were enrolled in the present study, and characteristics of **EML4-ALK** translocation, including the frequency, were determined in these NSCLC patients. **EML4-ALK** fusion variants were detected using Multiplex one-step reverse transcription-polymerase chain reaction and subsequently confirmed by DNA sequencing and Vysis ALK Break Apart fluorescence in situ hybridization analysis. The **EML4-ALK** fusion variants were detected in 21 carcinoma tissue specimens, accounting for 7.5% of the enrolled patients. Out of these patients with **EML4-ALK** fusion variants, **EML4-ALK** fusion variant 1 was identified in 12 patients, indicating that variant 1 is the most common type of **EML4-ALK** fusion gene in the present cohort of patients. ALK mRNA was abnormally expressed in all the tissues with **EML4-ALK** translocation, but not in the carcinoma tissues without **EML4-ALK** translocation. In addition, the **EML4-ALK** translocation was more frequently found in younger patients. The median age of patients with **EML4-ALK** translocation was 50.95±2.29 years, which was significantly younger (P<0.01) than the median age of the patients without **EML4-ALK** translocation (57.15±5.6). The **EML4-ALK** translocation was detected exclusively in undifferentiated tumors that were graded as poorly- or moderately-differentiated carcinomas and suspected to be more malignant compared with well-differentiated tumors. In summary, the present study found that 7.5% of patients with NSCLC that are female never-smokers harbor **EML4-ALK** translocations, which are associated with the aberrant expression of ALK mRNA, early onset of disease and undifferentiated carcinomas.

**Introduction**

Lung cancer is a devastating disease and the leading cause of cancer-associated mortality worldwide (1). The most frequent type of lung cancer is non-small-cell lung cancer (NSCLC), which accounts for ~80% of lung cancer cases (2). The short survival time of lung cancer patients is mainly attributed to poor outcomes from conventional chemotherapeutic treatments (3). However, progress in defining the molecular mechanism of carcinogenesis has led to a notable improvement in the response to chemotherapy (4). In 2004, it was revealed that epidermal
growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), including gefitinib and erlotinib, are only effective in patients that harbor tumorigenic EGFR mutations that cause aberrant tyrosine kinase activity (5,6). Thus, identification of oncogenic driver mutations in cancer patients has become key for the identification of an effective treatment for NSCLC (7). One of the previously identified oncogenic driver mutations is the fusion of anaplastic lymphoma kinase (ALK) with echinoderm microtubule-associated protein-like 4 (EML4) on chromosome 2p, which was identified in 2007 in a subpopulation of Japanese patients with NSCLC (8). In this study, 6.7% (5/75) of the enrolled Japanese patients with NSCLC possessed EML4-ALK fusion transcripts, resulting from ALK translocation within chromosome 2p (8). Other ALK-fusion genes, including KIF5B-ALK, have also been identified within chromosome 2p (9-12). Patients possessing ALK fusions are usually resistant to EGFR-TKIs (13), but respond to the ALK-TKI crizotinib (14). Therefore, screening for oncogenic driver mutations, including tumorigenic EGFR mutations and ALK fusions, has become a crucial step in disease diagnosis and designing an effective personalized or tailored therapy plan.

Since the identification of the EML4-ALK fusion gene, numerous studies have been performed to determine the frequency of occurrence in patients with NSCLC (8,12,15-24). However, these numbers varied significantly between studies (7), ranging between 1.6% in a cohort of Japanese patients (21) and 11.7% in a cohort of Chinese patients (22). This is likely to reflect the differences in detection techniques, sample size and patient selection criteria. Although the EML4-ALK translocation was first identified in a NSCLC patient with a history of smoking (8), subsequent studies have suggested that the translocation is more frequently detected in never-smokers (13,16,21,22). A never-smoker is defined as an individual that has smoked <100 cigarettes per lifetime, according to the US Center for Disease Control (25). Although inconclusive, studies have also suggested that the frequency of the incidence is likely to be increased in female patients compared with male patients (24). Thus, it is possible that the frequency of the EML4-ALK translocation may be markedly higher in female never-smokers. A previous study reported that the incidence was as high as 15.2% (5/33) in a small cohort of female patients with adenocarcinoma (24).

To determine the frequency of EML4-ALK fusion more precisely in female never-smokers, in the present study a large cohort of patients with NSCLC was assembled. In total, 280 female patients that were never-smokers were enrolled and the presence of mutations were detected by Multiplex one-step reverse transcription-polymerase chain reaction (RT-PCR) in the tumor specimens collected from these patients. The clinical characteristics that are associated with these mutations were also analyzed. The present study aimed to increase the understanding of the EML4-ALK fusion in NSCLC and provide information for improving the diagnosis procedure and designing personalized treatment plans.

Materials and methods

Patients and sample collection. The present study was approved by the Institutional Ethics Committee of Henan Cancer Hospital (Zhengzhou, China). In total, 280 never-smoking female patients with NSCLC were recruited (Table I). These patients were enrolled between 2012 and 2013 at Henan Cancer Hospital. Carcinoma tissue samples were collected from these patients and preserved as formalin-fixed paraffin-embedded (FFPE) tissue blocks. The FFPE tissue blocks were used as the only tissue sources for the experiments performed in the present study, including the detection of EML4-ALK fusions and measurement of the expression level of the ALK tyrosine kinase (ALK TK) mRNA and protein. As a standardized procedure during diagnosis, tumor subtypes and pathological characteristics were determined independently by two pathologists. The opinion of a third pathologist was required when there was a discrepancy. The tumors were graded based on the abnormality of the appearance of the tumor cells and tissues compared to the surrounding normal cells and normal tissues. Tumors that appeared close to normal were graded as well-differentiated. Tumors that appeared intermediate and highly abnormal were classified as moderately- and poorly-differentiated, respectively. The tumors that appeared to be abnormal were suspected to be fast-growing and malignant, collectively described as undifferentiated or less-differentiated tumors in the present study.

Detection of EML4-ALK fusion genes. The FFPE tissue blocks were sliced to a width of 3 µm, and the tumor regions were identified and collected for RNA extraction. Total RNA was extracted using RNeasy FFPE kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions, and treated with DNase I (DNA-free; Ambion Life Technologies, Carlsbad, CA, USA) to remove any DNA contamination. The RNA samples were then subjected to Multiplex One-step RT-PCR with fluorescent RT-PCR to detect EML4-ALK fusion transcripts using the human Lung Cancer Related Fusion Gene Detection kit (Yuanqi Bio-Pharmaceutical Co., Ltd., Shanghai, China), according to the manufacturer's instructions. Briefly, the mixture of each reaction contained 3 µl total RNA, 20 µl Multiplex RT-PCR buffer and 2 µl Multiplex Enzyme Mix in a

| Characteristics                  | Total, n (%) |
|----------------------------------|--------------|
| Histology                        |              |
| Adenocarcinoma                   | 274 (97.86)  |
| Squamous cell carcinoma          | 5 (1.79)     |
| Others                           | 1 (0.36)     |
| Age                              |              |
| <40 years                        | 4 (1.43)     |
| 40-49 years                      | 57 (20.36)   |
| 50-59 years                      | 105 (37.50)  |
| ≥60 years                        | 114 (40.71)  |
| Differentiation                  |              |
| Poorly-differentiated            | 91 (32.50)   |
| Moderately-differentiated        | 95 (33.93)   |
| Well-differentiated              | 94 (33.57)   |

Table I. Clinical features of non-small cell lung cancer tumors in female never-smokers.
total volume of 25 µl. The primers included in the reaction for the detection of EML4-ALK fusion subtype were as follows: V1 and V6 forward, 5'-ATTGGTGCAAGTGTGTTGC ATTG-3; V2 forward, 5'-CGGGAGACTATGAATATTG ACTG-3; V3a and V3b forward, 5'-ATGCATAATTCTTG GGGAA-3; V4b and V7 forward, 5'-GGGAAAAGGACCTA AAGGTTG-3; V4a forward, 5'-GTAAGCAGAGGAAAGG GCACTG-3; V5a and V5b forward, 5'-GCTAAAAGGCCC TTGGGCTG-3; and E17-A20 and V9 forward, 5'-CGTAC TCAATAGATGTCACCT-3; and the common reverse primer ALK-E20, 5'-CATGATGGTGCAAGGCTGTC-3' (Sangon Biotech Co., Ltd., Shanghai, China). RT-PCR was performed according to the manufacturer's instructions. Briefly, two DNA probes that targeted sequences prior to and following the ALK TK domain were designed for the ALK break apart FISH fusion transcripts. All patients expressing the EML4-ALK fusion, as determined by Multiplex RT-PCR and direct DNA sequencing, were further confirmed by Vysis ALK Break Apart fluorescence in situ hybridization (FISH) analysis.

**ALK break apart FISH analysis.** FISH experiments were performed using FFPE tissue sections in order to identify ALK rearrangements. The Vysis ALK Break Apart FISH Probe kit (Abbott Molecular Inc., Des Plaines, IL, USA) was used according to the manufacturer's instructions. Briefly, two DNA probes that targeted sequences prior to and following the ALK breaking point were labeled with green and red fluorescent dye, respectively, and used for FISH. In normal nuclei without ALK rearrangement, the red and green fluorescent signals from the two probes colocalize to form a yellowish signal, or the two signals are less than two signal diameters apart. However, when ALK rearrangement occurs, the signals from the two probes are located more than two signal diameters apart in a single nucleus, which indicates a cell with ALK rearrangement. A tumor sample was considered to lack ALK rearrangement if <5 cells out of 50 (<5/50 or <10%) appeared positive for ALK rearrangement. If >25 cells out of 50 (>25/50 or >50%) were positive for ALK rearrangement, the tumor was considered positive, but the tumor is equivocal if 5-25 cells (10 to 50%) are positive for ALK rearrangement. When the tumor was equivocal, additional evaluation was required, which was performed according to the manufacturer's instructions.

**Determining the level of ALK mRNA.** One-step RT-PCR was performed on the total RNA extracted from FFPE tissue blocks to determine the level of ALK RNA in the tissues. Primers specific to the ALK TK domain were designed for the ALK mRNA expression analysis. The expression level of the reference gene Abelson murine leukemia viral oncogene homolog (ABL) was also determined as a control. The primers used were as follows: ALK forward, 5'-AGAACCTGCTCC TTGACCT-3' and reverse, 5'-GGGCATCCACTCTAAGCTG C-3; and ABL forward, 5'-TACCTGAAAGGATGCTG ACC-3' and reverse, 5'-TTTCTTCTCCCGGTACCTCA-3'. In addition, the DNA sequencing primers were as follows: ALK sequencing primer, 5'-CCCTTTCTTATAGTAGCTCGCCCTG TAGAT-3; and ABL sequencing primer, 5'-CCATGTACACA GCAGACCCACGGGT-3'. RT-PCR was performed as aforementioned.

**Immunohistochemistry (IHC).** IHC was used to evaluate the expression of ALK TK protein, as previously described (26). Briefly, the experiments were performed on FFPE tissue sections using a Ventana ALK (D5F3) Cdx Assay (rabbit monoclonal antibody against ALK; cat no. 790-4796; Ventana Medical Systems, Inc., Tucson, AZ, USA), which detects the endogenous levels of total ALK protein and ALK fusion proteins, according to the manufacturer's instructions. The tissue sections were deparaffinized and incubated with 3% H2O2 (Ventana Medical Systems, Inc.) to quench endogenous peroxidase activity, which was followed by heat-induced antigen retrieval for 30-60 min (Ventana Medical Systems, Inc.). Subsequent to blocking with 10% normal goat serum (Ventana Medical Systems, Inc.), the aforementioned primary antibody was applied, followed by goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (ultraView Universal DAB Detection kit; cat no. 760-500; Ventana Medical Systems, Inc.), according to the manufacturer's instructions. The immunosignals were visualized using diaminobenzidine (UltraView; Ventana Medical Systems, Inc.) and counterstained with hematoxylin (Ventana Medical Systems, Inc.). Images were captured to assess the intensity of staining, as follows: 0, no staining; 1+, light staining; 2+, moderate staining; and 3+, strong staining. The distribution of ALK immunostaining was also determined. Any tissue specimens exhibiting IHC intensity >0 were defined as IHC positive.

**Statistical analysis.** The differences between data were analyzed using χ2 test, Mann-Whitney U test or Student's t-test, as appropriate. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Clinical features of participating patients.** To avoid the variation caused by a small sample size and to more precisely determine the frequency of EML4-ALK translocation, a total of 280 patients with NSCLC were recruited for the present study. All patients were female and were defined as never-smokers (25). Based on the information collected from these patients, the majority of the patients were diagnosed with adenocarcinoma (97.86%; Table I). The median age of this cohort of patients was 56.69 years, ranging between 23 and 76 years. Out of these patients, 40.71% of patients were aged >40 years, respectively (Table I). The number of patients with poorly, moderately or well-differentiated tumor cells was similar between the groups, with 91 (32.50%), 95 (33.93%) and 94 (33.57%) patients in each group, respectively (Table I).

**Detection and characterization of EML4-ALK translocation.** In the present study, the frequency of EML4-ALK translocation in female never-smokers with NSCLC was determined. Therefore, Multiplex one-step RT-PCR was performed on the total RNA extracted from the FFPE tissue samples prepared
translocation in female never-smokers with NSCLC. By breaking translocation, but not in carcinoma samples translocation in the harboring the fusion, (Fig. 3A). The identity of these RT-PCR translocation (median of the fusion gene tran of the mRNA was measured by one-step RT-PCR. The primers EML4-ALK translocation. Aberrant expression of ALK mRNA is associated with group of patients. The fusion products in female never-smokers with NSCLC. By all possible that detected detected in one sample each (Fig. 2B). Variant 2 was not detected, including variant 1 in 12 samples and variant 3a/3b in 6 samples. In addition, variants 4, 5a and E17-A20 were detected, including red and green fluorescent signals located at least two signal diameters apart in a single nucleus, were detected in carcinoma samples harboring EML4-ALK translocation, but not in carcinoma samples without EML4-ALK fusion. The identity of these RT-PCR products was confirmed by DNA sequencing. All RT-PCR products from 21 tissue samples harboring EML4-ALK translocation were subsequently sequenced. The DNA sequencing traces and exon organization for each representative variant are presented in Fig. 2A. In total, 5 different variants were detected, including variant 1 in 12 samples and variant 3a/3b in 6 samples. In addition, variants 4, 5a and E17-A20 were detected in one sample each (Fig. 2B). Variant 2 was not detected in any of the present tissue samples. Therefore, it is possible that EML4-ALK variant 1, which accounted for 57.1% of all EML4-ALK translocations, is the most common form of the fusion products in female never-smokers with NSCLC. By contrast, the variant 2 is less likely to be detected in the same group of patients.

Aberrant expression of ALK mRNA is associated with EML4-ALK translocation. The expression level of ALK mRNA was measured by one-step RT-PCR. The primers were specific to the ALK TK domain that was translocated and fused with EML4 in EML4-ALK fusion genes, so the mRNAs transcribed from the intact and translocated EML4 ALK genes would be detected. This method revealed that ALK TK mRNA was highly expressed in tissues harboring EML4-ALK fusion, but not in tissues without the EML4-ALK fusion (Fig. 3A). Despite expressing different variants of the EML4-ALK fusion gene, all carcinoma specimens harboring the fusion expressed ALK TK RNA aberrantly. By contrast, ALK TK mRNA was not detectable in the majority of the samples not harboring EML4-ALK translocation. IHC also revealed that the ALK protein is highly expressed in specimens harboring EML4-ALK fusion, but the protein was not expressed or was expressed at an extremely low level in carcinomas without EML4-ALK fusion (Fig. 3B).

EML4-ALK translocation is more frequently detected in younger patients and undifferentiated carcinomas. All patients harboring the EML4-ALK translocation in the present cohort of patients were diagnosed with adenocarcinoma (Table II). However, it is possible that the number of other types of carcinoma was too low for EML4-ALK fusion genes to be detected (6 out of 280 patients; 2.14%). Therefore, the frequency of EML4-ALK translocation in other types of carcinomas was unclear. In total, 50, 12.28, 7.62 and 3.51% of the patients aged <40, 40-49, 50-59 and ≥60 years, respectively, harbored EML4-ALK fusion genes (Table II). The distribution of the patients with EML4-ALK fusion in the four different age groups was significantly different from the distribution of patients without EML4-ALK fusion (P<0.0019; Table II). Furthermore, patients harboring EML4-ALK translocation (median age, 50.95±2.29 years) were significantly younger than patients without EML4-ALK translocation (median age, 50.95±2.29 years) were significantly younger than patients without EML4-ALK translocation (median age, 50.95±2.29 years) were significantly younger than patients without EML4-ALK translocation (median age, 50.95±2.29 years).
age, 57.15±0.56; P<0.01) and all patients combined (median age, 56.69±0.54; P<0.05) (Fig. 4). Histological examination revealed that all carcinomas harboring EML4-ALK fusion were undifferentiated, either poorly- or moderately-differentiated, but never well-differentiated (Table II). The difference in the differentiation level between carcinomas harboring EML4-ALK fusion and those without the fusion gene is significant (P<0.0014). Overall, these results indicate that the EML4-ALK fusion is more frequent in younger patients and in undifferentiated or less-differentiated carcinomas.

Discussion

By combining multiple published studies, the frequency of the EML4-ALK translocation in NSCLC worldwide was determined to be ~5% (7). In the present study, the frequency
of \(EML4-ALK\) fusion determined using Multiplex one-step RT-PCR was 7.5% (21 out of 280 patients) in a large cohort of never-smoking female patients with NSCLC. This result was verified by DNA sequencing, ALK Break Apart FISH analysis, and immunohistochemistry. This frequency is slightly increased compared with all NSCLC patients worldwide, regardless of gender and smoking history, indicating that the combined effect of gender (female) and smoking habit (never-smokers) is minor. Notably, the present results vary from the findings of a previous study that assessed 33 female never-smoker patients with NSCLC and reported that the incidence of \(EML4-ALK\) fusion was 15.2% (5 patients) (24). The possible discrepancy may be due to the variance caused by the difference in sample sizes between the two studies, with 33 female never-smoker patients in the previous study and 280 in the present study. In addition, environmental factors, such as local air pollution levels and food choices may vary between the different regions of China and may also be involved in the discrepancy.

Out of the 21 patients with \(EML4-ALK\) fusion genes, more than one-half were detected as fusion gene variant 1. Variant 2 was not detected in the present tissue samples. It is therefore possible that variant 1 is the predominant type, while variant 2 is the least likely to be detected in female never-smokers with NSCLC. Regardless of the variant present, the ALK mRNA and protein were aberrantly expressed in all \(EML4-ALK\)-positive carcinomas (Fig. 3). This result is consistent with the findings that \(EML4-ALK\) fusion genes are carcinoma driver mutations (8) and that overactivation of ALK TK plays a pivotal role in tumor cell proliferation (26-28). Compared with conventional chemotherapy, crizotinib, a selective inhibitor of ALK, has demonstrated a superior ability to improve the treatment outcome and survival rate in patients harboring \(EML4-ALK\) fusion mutations in clinical trials (26,29-32). By contrast, these patients demonstrated resistance to EGFR TKIs (13). Therefore, identification of \(EML4-ALK\) fusion has a direct impact on disease treatment in NSCLC, particularly with female never-smokers that demonstrate a high frequency of the \(EML4-ALK\) fusion.

In the present cohort of patients, \(EML4-ALK\) fusions were only detected in undifferentiated or less-differentiated...
carcinomas, including those graded as poorly- and moderately-differentiated tumors, but not in tumors graded as well-differentiated (Table II). This association between EML4-ALK fusion genes and undifferentiated carcinomas was also reported in a previous study, which was conducted using a cohort consisting of male and female patients with NSCLC (33). Since ALK is aberrantly expressed only in the carcinoma lesions harboring EML4-ALK gene fusion, but not in carcinomas without the fusion gene, it is possible that ALK expression plays an important role in determining the fate of cells in NSCLC and is accountable for decreased differentiation, a feature that is often associated with more rapid-growing and malignant types of carcinomas.

In the present study, the median age of patients with EML4-ALK gene fusion (50.95 years) was >6 years younger than the median age of patients without EML4-ALK gene fusion (57.15 years) in the present cohort of female never-smokers with NSCLC. The difference in age distribution between the patients harboring EML4-ALK gene fusion and those without gene fusion was statistically significant, as determined by χ² (P <0.05; Table II). In addition, the frequency of EML4-ALK gene fusion in the patients aged <40 years is 50%, while the frequency gradually decreases in the older age groups (Table II). In particular, only 3.5% of patients aged ≥60 years harbored the EML4-ALK fusion gene. These results suggest that the age of disease onset is significantly younger in patients harboring EML4-ALK gene fusion compared with patients that did not harbor gene fusion.

In conclusion, the frequency of the EML4-ALK fusion is 7.5% in a cohort of 280 NSCLC patients that consisted of female never-smokers. Among the identified fusion variants, variant 1 was the most common type, accounting for 57.1% of all EML4-ALK-positive cases. The mRNA and protein of ALK were aberrantly expressed in all EML4-ALK-positive carcinoma lesions, but were absent or expressed at a low level in patients without the fusion gene, suggesting an important role of EML4-ALK translocation in tumorigenesis. The EML4-ALK translocation is detected more frequently in younger patients and in undifferentiated carcinomas. These results may provide useful insights to the knowledge of NSCLC and facilitate the diagnosis and targeted treatment of the disease, leading to an improved treatment outcome and patient life quality.
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