Frequency, clinical features and differential response to therapy of concurrent ALK/EGFR alterations in Chinese lung cancer patients

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Purpose: EGFR and anaplastic lymphoma kinase (ALK) alterations have been regarded as oncogenic drivers and incorporated into clinical practices to manage nonsmall cell lung cancer (NSCLC). Alterations of these two genes were traditionally considered to be mutually exclusive, but recent studies have suggested that they can occur concomitantly. Here, we investigated the prevalence, clinical features and outcomes in response to the treatment of NSCLC patients who harbor EGFR and ALK co-alterations.

Methods: We reviewed the genomic profiles of 419 ALK-rearranged NSCLC patients with the intent of investigating the EGFR kinase domain (exon 18–21) and ALK co-alterations. The genomes of these patients were sequenced in a Clinical Laboratory Improvement Amendments-certified laboratory.

Results: The overall frequency of concomitant EGFR (exon 18–21) and ALK alterations was 5.01% (21/419) in ALK-rearranged NSCLC patients. The concomitant rate of EGFR alterations in patients with EML4-ALK co-alterations (3.06%, 11/359) was dramatically lower than that in patients with non-EML4-ALK co-alterations (16.67%, 10/60, p<0.01). EML4-ALK/EGFR co-alterations were more prone to occur in females than in males, and non-EML4-ALK/EGFR co-alterations were more common in males than in females (p=0.02). Before the detection of EGFR-ALK co-alterations, some patients were treated with EGFR-TKIs (n=16) according to previously detected EGFR alterations; Kaplan–Meier analysis revealed that EML4-ALK/EGFR co-altered patients (n=7) had a significantly shorter progression-free survival (PFS) after EGFR-TKI treatment than that of non-EML4-ALK/EGFR co-altered patients (n=8; mPFS, 6.0 vs 15.0 months, p=0.046). In addition, we demonstrated the subsequent clinical outcomes of co-altered patients after previous EGFR-TKI treatment. Five EGFR/ALK co-altered patients treated with single TKIs (EGFR-TKIs or ALK-TKIs) displayed diverse clinical outcomes. Three patients who received dual-TKI treatment (EGFR-TKI plus ALK-TKI) all achieved a PFS of more than 5 months (8.4 months, 8.6 months, >5.2 months).

Conclusion: EML4-ALK/EGFR and non-EML4-ALK/EGFR co-alterations displayed distinct clinical features and responses to EGFR-TKIs, suggesting that non-EML4-ALK co-alterations are likely to occur as a resistance mechanism to EGFR-TKI. In addition, dual-TKI therapy might be a better choice than single-TKI treatments for these co-altered patients. To the best of our knowledge, this is the largest dual-positive EGFR/ALK cohort study in People’s Republic of China.

Keywords: EGFR alteration, ALK rearrangement, nonsmall cell lung cancer, EML4-ALK, tyrosine kinase inhibitor
Introduction

EGFR and anaplastic lymphoma kinase (ALK) gene alterations have both been regarded as oncogenic driver mutations and drug targets in nonsmall cell lung cancers (NSCLCs).\(^1\)\(^-\)\(^3\) EGFR and ALK alterations were conventionally considered to be mutually exclusive\(^4\)\(^-\)\(^6\) and as mutual causes of resistance to ALK-TKIs or EGFR-TKIs.\(^7\)\(^,\)\(^8\) However, co-alterations of EGFR and ALK exist in a subset of NSCLCs and challenge the previous dogma.\(^9\)\(^-\)\(^11\)

In ALK-rearranged NSCLC patients, echinoderm microtubule-associated protein-like 4 (EML4) is the main fusion partner of ALK. EML4-ALK, in which the N-terminal of EML4 is fused to the intracellular kinase domain of ALK, displays constitutive ALK activation and generates oncogenic activity.\(^4\)\(^,\)\(^5\) In addition to EML4, other ALK 5′-partners have also been identified, including kinesin family member 5B, TRK-fused gene, and kinesin light chain 1.\(^12\)\(^-\)\(^14\) The frequency of non-EML4-ALK alterations is approximately 10–20% in ALK-positive lung cancers,\(^15\)\(^,\)\(^16\) and the clinical significance of these alterations still under investigation.

In previous studies about patients with EGFR and ALK co-alterations, researchers often combined patients with EGFR/ALK co-alterations as a single group, regardless of the ALK fusion partner, for clinical features or drug efficacy investigations. Little is known about the difference in clinical features and drug efficacy between the EML4-ALK/EGFR and non-EML4-ALK/EGFR co-alteration subgroups. Here, we interrogated the distinct concurrent alterations rate, clinical features, and clinical outcomes during EGFR-TKI treatment in both the EML4-ALK/EGFR and non-EML4-ALK/EGFR co-alteration subgroups. In addition, we sought to evaluate the clinical activity of these co-altered patients in response to single-TKI or dual-TKI treatments.

Materials and methods

Patient information

We retrospectively reviewed the genomic profiling data of 7,661 lung cancer patients, whose tissue or plasma samples were sequenced in a Clinical Laboratory Improvement Amendments-certified clinical molecular diagnostic laboratory using next-generation sequencing (NGS) between September 2015 and January 2018. Among them, 419 patients were identified as harboring ALK rearrangements. The clinical characteristics of patients harboring dual-positive EGFR and ALK alterations were collected. All patients had a histologically confirmed diagnosis of advanced-stage NSCLC. Progression-free survival (PFS) after EGFR-TKI treatment and survival information were assessed for the cohorts. The study was approved by the institutional review board of Peking University Shenzhen Hospital. All other centers were covered by this protocol. All patients whose tissue and medical data were used in this research provided written informed consent, in accordance with the Declaration of Helsinki.

Tissue DNA and plasma cfDNA preparation

The tissue DNA was extracted from all tissue samples using the QIAamp DNA FFPE tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Circulating cfDNA was recovered from 4 to 5 mL plasma by using the QIAamp Circulating Nucleic Acid kit (Qiagen). DNA was quantified with the Qubit 2.0 fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA).

Targeted DNA sequencing

The genomic DNA was profiled by using capture-based targeted sequencing panel that consisted of 8, 56, 168 or 295 cancer-related genes (Burning Rock Biotech, Guangzhou, People's Republic of China). Alterations of eight well-established driver genes, EGFR, ERBB2, ALK, ROS1, RET, KRAS, BRAF and MET, were all included in these sequencing panels. The NGS library was prepared as previously described.\(^17\) In brief, DNA was sheared using Covaris M220 (Covaris, Inc., Woburn, MA, USA), which was then followed by end-repair, phosphorylation and adaptor ligation. Fragments that were 200–400 bp in size were selected by beads (Agencourt AMPure XP Kit, Beckman Coulter, Brea, CA, USA), followed by hybridization with capture probes or baits, hybrid selection with magnetic beads and PCR amplification. A high-sensitivity DNA assay was performed using a bioanalyzer to evaluate the DNA quality and size. Indexed samples were then sequenced on a NextSeq 500 (Illumina, Inc., San Diego, CA, USA) with pair-end reads.

The sequencing data were in a FASTQ format and mapped to the human genome (hg19) using BWA aligner 0.7.10.\(^18\)\(^,\)\(^19\) Local alignment optimization, mark duplication and variant calling were performed using the Genome Analysis ToolKit 3.2,\(^20\) Picard (http://picard.sourceforge.net) and VarScan.\(^21\) Gene translocations were called with FACTERA,\(^22\) and the copy number variation was analyzed with an in-house algorithm based on sequencing depth.
Statistical analysis

Statistical analysis was conducted using R software version 3.3.3. Between the EML4-ALK and non-EML4-ALK subgroups, differences in sex and mutation rate were calculated and presented using Fisher’s exact tests, while differences in age were calculated using paired, two-tailed Student’s t-tests. For all statistical tests, \( p < 0.05 \) was considered statistically significant.

Results

Patient characteristics

The genomic sequencing results of 419 ALK-rearranged NSCLC patients were retrospectively reviewed, including 359 (85.7%) EML4-ALK fusions and 60 (14.3%) non-EML4-ALK fusions. Among the 419 ALK-rearranged lung cancers, a total of 21 patients (5.01%) were detected to harbor concurrent ALK and EGFR (exon 18–21) genomic alterations. The concomitant rate of EGFR alterations in patients harboring EML4-ALK co-alterations (3.06%, 11/359) was dramatically lower than that in non-EML4-ALK co-altered patients (16.67%, 10/60, \( p < 0.01 \), Fisher’s exact test, Table 1).

All the 21 ALK/EGFR co-altered cases were diagnosed as adenocarcinomas. In the EML4-ALK/EGFR co-altered subgroup, 4 (36%) patients were male, and 7 (64%) patients were female. In contrast, the non-EML4-ALK/EGFR co-altered subgroup comprised 9 (90%) males and 1 (10%) female (\( p = 0.02 \), Fisher’s exact test). This difference suggested that EML4-ALK/EGFR co-alterations were more prone to occur in females than males, and non-EML4-ALK/EGFR co-alterations were more common in males than in females. The median age of the EML4-ALK/EGFR and non-EML4-ALK/EGFR co-altered subgroups were 53.0 and 59.5 years, respectively (\( p = 0.31 \), t-test). The patient characteristics are summarized in Table 1.

Identification of molecular patterns of co-altered ALK/EGFR

In the 11 EML4-ALK/EGFR co-altered patients, capture-based sequencing identified different EML4-ALK variants, including 6 with E13A20 (V1), 3 with E6A20 (V3), and 2 with E2A20 (Figure 1). As for the 10 non-EML4-ALK/EGFR co-altered patients, 6 unique ALK fusion partners were detected. STRN was the most common fusion partner in non-EML4-ALK co-alterations and was identified in five patients (50%). Apart from those five patients, another two STRN-ALK positive patients were identified in the whole cohort of 419 ALK-rearranged NSCLC patients. One patient harbored a co-alteration of STRN-ALK and EGFR exon 15 V592I, and another patient was identified as being STRN-ALK-positive only. This observation suggested that co-alterations with EGFR were a common feature of STRN-ALK fusions. Before these five patients were detected to have STRN-ALK/EGFR (exon 18–21) co-alterations, all of them had previously been detected to have EGFR alterations and had been treated with EGFR-TKIs but not with ALK-TKIs, indicating that STRN-ALK might be one of the resistance mechanisms for EGFR-TKIs. In addition, five novel ALK fusion partners were identified including WDR37-ALK, CEBPZ-ALK, KIDINS220-ALK, LOC284950-ALK and MFSD2B-ALK.

Of the 21 patients who harbored co-altered ALK/EGFR, the following 11 patients with EML4-ALK/EGFR co-alterations were identified to have diverse EGFR variants: 7 patients had exon 19 deletions, 2 had exon 21 L858R point mutations, 1 had a P753S point mutation and 1 had a G863D point mutation. Meanwhile, the 10 non-EML4-ALK/EGFR-altered patients included 6 EGFR exon 19 deletions, 3 L858R point mutations and 1 V769M point mutation (Figure 2).

Is concomitant non-EML4-ALK co-alterations a de novo or acquired rearrangement?

The distribution of EML4-ALK and non-EML4-ALK co-alterations in the “de novo ALK”, “acquired ALK” and “uncertain” groups are summarized in Table 2. Patients identified with co-altered EGFR/ALK at the time of the first diagnosis were classified as “de novo ALK” since they had not received TKI treatment before. This group contained 4 patients (4/5, 80%) with EGFR/EML4-ALK co-alterations and 1 patient (1/5, 20%) with an EGFR/non-EML4-ALK co-alteration. Patients who were identified as EGFR (+)/ALK (-) at a previous diagnosis and later detected to have co-altered EGFR/ALK after developing EGFR-TKI resistance were classified as “acquired ALK”; this group consisted of 1 patient with (1/4, 25%) an EGFR/EML4-ALK co-alteration and 3 patients (3/4, 75%) with EGFR/non-EML4-ALK co-alterations. In addition, patients who were detected to have EGFR/ALK co-alterations after developing EGFR-TKI resistance but whose previous ALK status was unavailable were defined as “uncertain”; this group included 6 patients with EGFR/EML4-ALK co-alterations and 6 patients with EGFR/non-EML4-ALK co-alterations. The observation that EML4-ALK co-alterations accounted for most of the rearrangements in the de novo ALK patients (80%) while non-EML4-ALK co-alterations accounted for most of the alterations in the acquired ALK patients (75%)
after EGFR-TKI treatment indicated that EML4-ALK co-alterations were likely to be de novo alterations and non-EML4-ALK co-alterations might be likely to appear as a drug resistance mechanism of EGFR-TKIs.

Next, for patients progressed on previous EGFR-TKI treatment ("acquired ALK" plus "uncertain" subgroups), we interrogated the correlation of PFS in response to EGFR-TKIs before the detection of ALK rearrangements and the distribution of ALK fusion partners after EGFR-TKI treatment. The Kaplan–Meier analysis revealed that after EGFR-TKI treatment, patients with EML4-ALK/EGFR co-alterations (n=7) commonly had a significantly shorter median PFS than that of patients with non-EML4-ALK/EGFR co-alterations (n=8; PFS information of one patient was unavailable; mPFS, 6.0 vs 15.0 months, p=0.046, Figure 3). The discrepancy in PFS between the two subgroups has never been reported before and could suggest that EML4-ALK co-alterations were likely to be induced in the early stages of EGFR-TKI treatment, whereas non-EML4-ALK co-alterations might be acquired at relatively late stages after EGFR-TKI treatment.

Subsequent TKI efficacy after previous EGFR-TKI treatment

The clinical data of eight patients with EGFR/ALK co-alterations who received subsequent single- or dual-TKI treatment after already receiving previous EGFR-TKI treatment were collected and are presented in Figure 4. Of the eight cases, six (75%) of them were detected to have the first-generation EGFR-TKI resistant mutation T790M, indicating that ALK rearrangements might occur concomitantly with EGFR-T790M after EGFR-TKI therapy. Five patients were treated with subsequent single TKIs (EGFR-TKI or ALK-TKI) after the detection of EGFR/ALK co-alterations, and the clinical efficacy of the single TKI varied greatly. Among those with the T90M mutation, three patients who received subsequent osimertinib treatment had a PFS of 2.5, 2.9 and >10.6 months. One patient received combined afatinib and

Table 1 Characteristics of patients harboring dual-positive ALK/EGFR alterations

| Patient Characteristics | EML4-ALK | Non-EML4-ALK | P-value |
|-------------------------|----------|--------------|---------|
| Total                   | 11       | 10           |         |
| Co-occurrence rate in ALK-rearranged NSCLC | 3.06% (11/359) | 16.67% (10/60) | p<0.01 |
| Gender                  |          |              |         |
| Male                    | 4        | 9            | p=0.02  |
| Female                  | 7        | 1            | p=0.31  |
| Age (year)              | 53       | 59.5         |         |
| Median                  | 42–74    | 44–81        |         |
| Range                   | 11       | 10           |         |
| Histological types      |          |              |         |
| Adenocarcinoma          | 11       | 10           |         |

**Abbreviations:** ALK, anaplastic lymphoma kinase; NSCLC, nonsmall cell lung cancer; EML4, echinoderm microtubule-associated protein-like 4.

Figure 1 Structure and breakpoints of the 21 ALK fusions in EGFR-mutated nonsmall cell lung cancer patients identified by next-generation sequencing. E2:E20 indicates that exon 2 of EML4 is fused to exon 20 of ALK. The number in the brackets indicates the number of patients with that respective fusion variant.

**Abbreviations:** ALK, anaplastic lymphoma kinase; EML4, echinoderm microtubule-associated protein-like 4.
osimertinib and had a PFS of more than 13.6 months. One patient was treated with osimertinib plus chemotherapy and only had a PFS of 1.9 months, and one patient received crizotinib and achieved a PFS of 3.7 months. In addition, three patients received subsequent dual-TKI treatment (EGFR-TKI plus ALK-TKI), and the PFS of all three patients were longer than 5 months (8.4 months, 8.6 months, >5.2 months), indicating that patients might benefit from the combination therapy of ALK-TKIs and EGFR-TKIs.

**Discussion**

*EGFR* mutations and *ALK* rearrangements were commonly considered to be mutually exclusive in previous studies. However, there is increasing evidence to support the notion that they can be concomitantly mutated in cancers. In this study, we retrospectively reviewed the genomic profiling data of 419 *ALK*-positive Chinese NSCLC patients and identified 21 patients who harbored concurrent *EGFR* and *ALK* gene alterations. We investigated and compared the prevalence, clinical features, and different responses to tyrosine kinase inhibitors in subgroups of patients harbored *EML4-ALK/EGFR* or non-*EML4-ALK/EGFR* co-alterations. We found that *EML4-ALK/EGFR* and non-*EML4-ALK/EGFR* co-alterations showed different clinical characteristics and clinical outcomes to EGFR-TKIs, suggesting that non-*EML4-ALK* co-alterations might be likely to emerge as a drug resistance mechanism for EGFR-TKIs. In addition, dual-TKI therapy might be a better choice for patients with co-altered *ALK/EGFR* than single-TKI therapy. To the best of our knowledge, this is the largest cohort study of *ALK* rearrangements for dual *EGFR/ALK* co-alterations in People's Republic of China. In addition, this is the largest series of *ALK*-positive patients reported, and five novel *ALK* fusion partners were identified in this study.

There were inconsistencies about the clinical features of *EGFR/ALK* co-mutated patients. Yang et al reported that the median age in *EGFR/ALK* co-altered patients was 59 years, and the number of patients who were female and male were 8 (62%) and 5 (38%), respectively. A European study revealed that there were two males and one female in their study of three *EGFR/ALK* co-altered patients. We also interrogated the clinical demographics of *EGFR/ALK* comutated patients in this study. We found that the median age of the co-altered patients was 58 years old. Among them, 8 (38.1%) patients were female and 13 (61.9%) were male. This difference in sex was probably induced by different factors and the limited cohort size of each study. Moreover, none of the previous studies compared the clinical characteristics between patients

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**Table 2** Distribution of *EML4-ALK* and non-*EML4-ALK* in de novo and acquired *ALK* rearrangements

| ALK fusions     | NGS before baseline* | Treatment before baseline | EML4-ALK | Non-EML4-ALK |
|-----------------|----------------------|---------------------------|----------|--------------|
| De novo ALK (n=5) | N/A                  | Treatment-naive           | 4/5 (80%)| 1/5 (20%)    |
| Acquired ALK (n=4) | EGFR (+), ALK (-)   | EGFR-TKI                  | 1/4 (25%)| 3/4 (75%)    |
| Uncertain (n=12)  | EGFR (+), ALK (unknown) | EGFR-TKI | 6/12 (50%) | 6/12 (50%) |

Notes: *The timepoint of positive-detection of co-altered *EGFR/ALK* was defined as baseline.*

Abbreviations: *ALK*, anaplastic lymphoma kinase; N/A, not applicable; NGS, next generation sequencing; *EML4*, echinoderm microtubule-associated protein-like 4.
with EML4-ALK/EGFR and non-EML4-ALK/EGFR co-alterations. We found that EML4-ALK/EGFR co-alterations were more prone to occur in females than in males, and non-EML4-ALK/EGFR co-alterations were more likely to occur in males than in females (p=0.02). The median age of EML4-ALK/EGFR co-altered patients was slightly younger than that of non-EML4-ALK/EGFR co-altered patients (53.0 vs 59.5 years, p=0.31). This clinically relevant observation may be important for further investigations of treatment strategies.

Several previous studies have reported that acquired ALK rearrangements could occur as a resistance mechanism after EGFR-TKI treatment. For example, Liang et al reported that ALK fusions were detected in the new metastatic lesion of an EGFR-mutated NSCLC patient.\(^8\) In our study, we found that after EGFR-TKI treatments EML4-ALK co-alterations accounted for most of the 5 de novo ALK-rearranged patients (80%, 4/5), while the non-EML4-ALK co-alterations accounted for most of the 4 acquired ALK-positive patients (75%, 3/4). We also observed that EML4-ALK/EGFR co-altered patients had a significantly inferior median PFS in response to EGFR-TKI compared to that of non-EML4-ALK/EGFR co-altered patients (mPFS, 6.0 vs 15.0 months, p=0.046). These results indicated that EML4-ALK co-alterations were likely to be a de novo alteration and non-EML4-ALK co-alterations might be an acquired resistance mechanism induced by EGFR-TKIs. Moreover, EML4-ALK co-alterations were likely to be induced in the early stages during EGFR-TKI treatment, and non-EML4-ALK co-alterations might emerge at a relatively late stage. Although the evidence of non-EML4/ALK co-alterations as a resistance mechanism to EGFR-TKIs has not been solidified, our study has greatly expanded the current knowledge of the underlying association of EGFR and ALK alterations and might be helpful for understanding the co-alteration mechanism and guiding clinical decision-making. In addition, we proposed that for patients developing first-line EGFR-TKI treatment resistance, genomic profiling and sequencing analysis should be performed rather than

Figure 3 Kaplan–Meier curve demonstrates the correlation of PFS in response to EGFR-TKIs before ALK detection and the distribution of ALK fusion partners after EGFR-TKI treatment. The risk table (below) illustrates the number of patients included per time point.

**Abbreviations:** ALK, anaplastic lymphoma kinase; EML4, echinoderm microtubule-associated protein-like 4; PFS, progression-free survival.
testing for \textit{EGFR}-T790M solely since the \textit{EGFR}-T790M mutation and \textit{ALK} rearrangements may be detected simultaneously.

A limited number of studies have reported about the clinical efficacy of TKIs in patients who harbor \textit{EGFR/ALK} co-alterations and have reached diverse conclusions. A Caucasian patient with lung adenosquamous carcinoma who harbored such a co-alteration displayed resistance to erlotinib treatment.\cite{24} Ten NSCLC patients in a Chinese cohort achieved a response rate of 80\% to the first-line EGFR-TKIs.\cite{9} In a Korean cohort with 14 co-altered patients, 3 patients who were treated with gefitinib showed poor response, and 8 patients who received \textit{ALK} inhibitors showed good response with a response rate of 87.5\%.\cite{11} Previous studies with targeted therapy on \textit{EGFR/ALK} co-altered patients commonly focused on single TKIs or the sequential use of \textit{EGFR}- and \textit{ALK}-TKIs. No studies have compared the clinical outcomes of single TKIs with combinatorial therapies of \textit{EGFR}-TKIs and \textit{ALK}-TKIs in \textit{EGFR/ALK} co-altered patients. Here, we demonstrated the clinical activities of single TKIs and dual TKIs in \textit{EGFR/ALK} co-altered NSCLC patients who progressed after EGFR-TKIs. We found that the clinical efficacy of single TKIs in five patients varied greatly. We also observed that patients who harbor dual alterations might benefit from the combination therapy of \textit{ALK}-TKIs and EGFR-TKIs, and the PFS of all three cases with dual alterations in this study were longer than 5 months.

However, there were several limitations in this study. First, 21 (1.2\%) NSCLC patients were identified to have \textit{EGFR/ALK} co-alterations from a total of 419 \textit{ALK}-positive patients. Based on the small number of patients, it was difficult to draw clear conclusions on the clinical characteristics and therapeutic outcomes of the patients. Second, since not all samples were collected at baseline and since there was previously identified targetable mutations and treatment records were lacking, we proposed several concepts from our analysis, and further investigations are still needed to validate our ideas.

\textbf{Conclusion}

In summary, we demonstrated that NSCLC patients with \textit{EML4-ALK/EGFR} and non-\textit{EML4-ALK/EGFR} co-alterations displayed distinct rates of concurrent alterations, clinical demographics and survival in response to EGFR-TKI treatment. We proposed that non-\textit{EML4-ALK} alterations are likely to be an acquired resistance mechanism later during EGFR-TKI treatment. The underlying
molecular mechanisms of the two alterations and potential of combination treatments with dual TKIs require further investigation with this co-altered subgroup.

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

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