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Retrospective Study**Coexistence of anaplastic lymphoma kinase rearrangement in lung adenocarcinoma harbouring epidermal growth factor receptor mutation: A single-center retrospective study**

Zhong WX *et al.* Coexistence of EGFR and ALK mutations

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Abstract**BACKGROUND**

Accumulating evidences confirm that epidermal growth factor receptor (EGFR) mutation and anaplastic lymphoma kinase (ALK) rearrangement have coexisted in lung adenocarcinoma (LUAD). However, Its biological mechanism, clinicopathological features, and optimization of targeted drugs have not yet been completely elucidated.

AIM

This study aimed to explore the clinical profile of LUAD patients with co-mutations of *EGFR* and *ALK* genes, with hopes of scientifically guiding similar patients towards selected, targeted drugs.

METHODS

Two hundred and thirty-seven LUAD patients were enrolled. EGFR Mutations were detected by the amplification refractory mutation system-peptide nucleic acid technique, while the expression of ALK rearrangement was screened by the 5'/3'

imbalance strategy for reverse transcription followed by quantitative polymerase chain reaction analysis. The clinicopathological features of these patients were analysed retrospectively, and the follow-up data were collected.

RESULTS

There were six cases with co-mutations of *EGFR* and *ALK* genes, which were more common in women, non-smoking and stage IV LUAD patients with bone metastasis, hence a positive rate of 2.53% (6/237). EGFR-tyrosine kinase inhibitors (EGFR-TKIs) were their preferred drugs for targeted therapy in these patients, with progression-free survival ranging from two months to six months.

CONCLUSION

In Gannan region, the positive rate of co-mutations of *EGFR* and *ALK* genes in LUAD patients is relatively high, and the co-mutations are more common in women, non-smoking and stage IV patients with bone metastasis. These patients prefer EGFR-TKIs as their preferred targeted drugs, but the therapeutic effect is not good. EGFR/*ALK* dual-TKIs may be more effective targeted drugs, which needs further study.

Key Words: Lung adenocarcinoma; Epidermal growth factor receptor mutation; Anaplastic lymphoma kinase rearrangement; Co-mutation; Tyrosine kinase inhibitor

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Core Tip: This study retrospectively analyzed the clinicopathological features of patients with co-mutations of *EGFR* and *ALK* genes in lung adenocarcinoma, and collected follow-up data of these patients, especially focusing on the tyrosine kinase inhibitors (TKIs) selected and its therapeutic effect in the real world from a single

institute experience. The results showed that the positive rate of lung adenocarcinoma patients with co-mutations of *EGFR* and *ALK* genes, which were more common in women, non-smoking and stage IV patients with bone metastasis, was relatively high in Gannan region, which may be related to regional heterogeneity. EGFR-TKIs were their preferred drugs for targeted therapy in these patients, but the therapeutic effect is not good. EGFR/*ALK* dual-TKIs may be more effective targeted drugs.

INTRODUCTION

With the availability of targeted drugs in non-small cell lung cancer (NSCLC) and the development of molecular detection technology, NSCLC has entered the era of precision medicine. Targeted therapy has brought revolutionary changes to NSCLC patients with epidermal growth factor receptor (EGFR) mutation or anaplastic lymphoma kinase (ALK) rearrangement. EGFR mutation is the most common and important therapeutic target, which is significantly associated with the sensitivity of EGFR-tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib, erlotinib and osimertinib. At present, EGFR-TKIs are widely used in clinical practice and have exhibited favourable anti-tumour effect in the treatment of NSCLC^[1,2]. ALK rearrangement is the second target after EGFR mutation, and it is also the first target of fusion gene tyrosine kinase inhibitor found in NSCLC. Targeted drugs for ALK rearrangement have developed rapidly, and the clinical use of ALK-TKIs, including crizotinib^[3], alectinib^[4], and lorlatinib^[5], has significantly improved the survival of advanced NSCLC patients with ALK rearrangement, even better than the efficacy of EGFR-TKIs.

Previous studies^[6] have indicated that EGFR mutation and ALK rearrangement are independent molecular events in NSCLC. However, Sporadic cases with Concomitance of EGFR mutation and ALK rearrangement have been increasingly reported^[7-10]. The biological mechanism, clinicopathological features and optimal targeted drugs of NSCLC patients with co-mutations of *EGFR* and *ALK* genes remain mostly unknown.

In this study, we investigated the clinicopathological features and follow-up data of patients with co-mutations of *EGFR* and *ALK* genes in lung adenocarcinoma (LUAD) within Gannan region, aiming to obtain the clinical profile of LUAD patients with co-mutations of *EGFR* and *ALK* genes, with intention to scientifically guide the selection of targeted drugs in similar patients. In the experiment, *EGFR* mutation was detected using amplification refractory mutation system-peptide nucleic acid (ARMS-PNA), and *ALK* rearrangement was screened by 5'/3' imbalance strategy for reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR).

MATERIALS AND METHODS

Patients

Two hundred and thirty-seven patients with primary LUAD confirmed by pathological examination in the First Affiliated Hospital of Gannan Medical University from 2016 to 2020 were enrolled, comprising 132 males (55.70%) and 105 females (44.30%); the average age was (60.3 ± 10.569) years, and the median age was 61 years (range 34-89); 145 cases (61.18%) had no smoking history and 92 cases (38.82%) had smoking history, which was defined as a cumulative minimum of 100 cigarettes. The clinical stage was obtained according to the International Association for the study of lung cancer (IASLC) TNM staging standard, eighth edition. There were 42 cases in stage I (17.72%), 16 cases in stage II (6.75%), 21 cases in stage III (8.86%) and 158 cases in stage IV (66.67%). These patients all came from Gannan region, Jiangxi province, and they were all untreated and had never received chemotherapy, radiotherapy, molecular targeted therapy or immunotherapy.

The data used in this study were all anonymous, which were not involved in the patients' privacy information, they were obtained after each patient agreed to treatment by written consent. This study have been approved by the Scientific Research Ethics Committee of the First Affiliated Hospital of Gannan Medical University (No. LLSC-2021081601), and conducted in accordance with Declaration of Helsinki (as revised in 2013).

Tissue samples

There were 237 tissue samples, including surgical resection tissue, computed tomography (CT) guided percutaneous lung biopsy tissue, and electronic bronchoscopy biopsy tissue. 200 tissue samples were embedded and fixed in paraffin by the Department of Pathology of the hospital, and 15 pathological white films were cut according to a thickness of at least 5 μm and sent out; 37 fresh tissue samples were stored in RNAfixer tissue preservation solution *in vitro* and sent out after fixation. All tissue samples were sent to Dingjing Medical Laboratory for genetic testing.

EGFR mutation detected by ARMS-PNA

DNA Extraction: A QIAamp DNA FFPE Extraction Kit (Qiagen) was used to extract the DNA of samples, while the purity and concentration of DNA were detected by spectrophotometer.

ARMS-PNA: Mutations in exons 18, 19, 20 and 21 of the *EGFR* gene were detected by ARMS-PNA technology. For the specific operation method, a human *EGFR* gene mutation detection kit (fluorescence PCR method) was used (Haijili, Registration Certificate No: 3400973).

Expression of ALK rearrangement detected by RT-qPCR with 5'/3' unbalanced strategy

Total RNA extraction: The total RNA of the samples was extracted by a tissue RNA Extraction Kit (Qiagen), and the concentration ($> 30 \text{ ng}$) and purity (OD260/OD280 value of 1.8-2.0) of total RNA in the template were determined by UV spectrophotometer.

Synthesis of complementary DNA by RT: The RT procedure was followed according to the instructions of M-MLV reverse transcription Kit: 25 °C for 5 min, 42 °C for 60 min, 75 °C for 5 min, and 4 °C for 5 min.

Real-time PCR system: RT products were prepared for PCR by using SYBR Green PCR Master Mix (Applied Biosystems) reagent. Fluorescent substances were added to the reaction system and detected by an ABI7500 PCR instrument (Thermo Fisher Scientific). According to the user manual of the human EML4-ALK fusion gene detection kit (real-time PCR), the PCR amplification was carried out under the following conditions: (1) Ung enzyme reaction: 1 cycle at 50 °C for 2 min; (2) Predenaturation: 1 cycle at 95 °C for 10 min; (3) Denaturation: 95 °C 15 s; annealing, extension and fluorescence detection (FAM as fluorescence channel) at 60 °C for 32 s; a total of 45 cycles; and (4) Dissolution curve (60 °C-95 °C increases by 1%, and the fluorescence signal is absorbed at this stage): 1 cycle at 95 °C for 15 s; 1 cycle at 60 °C for 1 min; 1 cycle at 95 °C for 30 s; 1 cycle at 60 °C for 15 s. The primer sequences were as follows: EML4-ALK-M (ALK-21/22F1: GCAA GTGGCTGTGAAGAC; ALK-22/23R1: GGTGGTTGAATTTGCTGATG), EML4-ALK-N (ALK-18F1: AAGGCCACGGGGAAGTG; ALK-18/19R1: GGGTGGGTGACACAATGC); GAPDH-Q (GAPDH-QF: GCCACATCGCTCAGACACC; GAPDH-QR: GATGGCAACAATATCCACTTTACC).

Statistical analysis

The data in this article are count data, expressed as percentage.

RESULTS

Co-mutations of EGFR and ALK genes

There were six cases of co-mutations of EGFR and ALK genes, and the positive rate was 2.53% (6/237) (Figure 1 A-E and Figure 2). One hundred and twenty cases with EGFR mutations were detected, and the mutation rate was 50.63% (120/237); twenty-one cases of ALK rearrangement were expressed, and the expression rate was 8.86% (21/237).

Clinicopathological features of LUAD patients with co-mutations of EGFR and ALK genes

The co-mutations of *EGFR* and *ALK* genes in patients with LUAD in Gannan region were more common in women, non-smokers and stage IV patients with bone metastasis. 19del mutation and L858R mutation were the main subtypes of *EGFR* mutation (Table 1).

¹*Clinical follow-up data of LUAD patients with co-mutations of EGFR and ALK genes*
EGFR-TKIs were their preferred drugs for targeted therapy for LUAD patients with co-mutations of *EGFR* and *ALK* genes in Gannan region, and the progression-free survival (PFS) ranged from two months to six months (Table 2).

DISCUSSION

EGFR mutation or *ALK* rearrangement has become a predictor of therapeutic sensitivity of *EGFR*-TKIs or *ALK*-TKIs in NSCLC patients. Accurate detection of *EGFR* mutation or *ALK* rearrangement can guide the selection of targeted drugs and ultimately realize individualized therapy. Early study^[11] demonstrated that co-mutations of *EGFR* and *ALK* genes were rare in NSCLC, and approximately 94% of *ALK* rearrangement cases were reported in *EGFR* wild type NSCLC.

However, a growing number of studies have confirmed that *EGFR* mutation and *ALK* rearrangement coexist in NSCLC and are increasingly reported. Wang *et al*^[12] summarized 66 patients with coexistence of *EGFR* mutation and *ALK* rearrangement, and displayed that the incidence of co-mutations of *EGFR* and *ALK* genes, which were more common in East Asian women, non-smoking and stage IV LUAD, was approximately 1%. In our study, six cases with co-mutations of *EGFR* and *ALK* genes were detected, with a higher positive rate of 2.53% (6/237), which may be related to regional heterogeneity. Liu *et al*^[13] found that the frequency of co-mutations of *EGFR* and *ALK* genes was as high as 5%, and proposed that more cases with co-mutations of *EGFR* and *ALK* genes identified were attributed to the utility of next-generation sequencing (NGS) technology. Our retrospective analysis indicated that co-mutations of

EGFR and *ALK* genes in Gannan region were more common in women, non-smokers and stage IV LUAD with bone metastasis.

Despite the co-mutations of *EGFR* and *ALK* genes in NSCLC have been gradually founded, its biological mechanism have not been exhaustively described. There are two hypotheses about the biological mechanism of co-mutations: one is the hypothesis of polyclonal origin, whereby there is heterogeneity between tumours^[14], and there may be two tumour cells carrying *EGFR* mutations or *ALK* rearrangements in the same tumour tissue; the other is the hypothesis of monoclonal origin^[15], wherein tumour cells carry *EGFR* mutations and *ALK* rearrangements at the same time.

To the best of our knowledge, targeted therapy is recommended for advanced NSCLC patients with specific oncogenic drivers such as *EGFR*, *ALK*. How to select the optimal targeted drugs for NSCLC patients with co-mutations of *EGFR* and *ALK* genes? So far, there is no guideline or consensus on the best treatment strategy for patients with coexistence of *EGFR* mutation and *ALK* rearrangement. In clinical practice, *EGFR*-TKIs are the indispensable drugs for first-line treatment of advanced NSCLC patients with concomitant *EGFR* mutation and *ALK* rearrangement, and the sequential use of *ALK*-TKIs is more common after disease progression. Yin *et al*^[16] reviewed the clinical efficacy of *EGFR*-TKIs and *ALK*-TKIs in 22 NSCLC patients with *EGFR*/*EML4*-*ALK* co-mutations, and concluded that *EGFR*-TKIs were the basic treatment for advanced NSCLC patients with concomitant *EGFR* mutation and *ALK* rearrangement; Shin *et al*^[17] reported that *EGFR*-TKIs were more effective than *ALK*-TKIs in three NSCLC patients with co-mutations of *EGFR* and *ALK* genes. Zhao *et al*^[9] found that both *EGFR*-TKIs and *ALK*-TKIs were effective in NSCLC patients with co-mutations of *EGFR* and *ALK* genes, and sequential use of *EGFR*-TKIs and crizotinib was a feasible alternative treatment strategy. In contrast, several studies^[14,18] shows that NSCLC patients with co-mutations of *EGFR* and *ALK* genes have a better response to crizotinib than *EGFR*-TKIs. A recent case study by Yin *et al*^[16] showed that a female LUAD patient with brain metastases harboured *EGFR* mutation and *ALK* rearrangement, and responded to successive osimertinib and alectinib treatment. Surprisingly, alectinib achieved an almost complete

response for lung and brain lesions after developing osimertinib resistance, providing a new perspective for the treatment of advanced NSCLC patients with concomitant EGFR mutation and ALK rearrangement. In addition, Mohapatra *et al*^[19] pointed out that, owing to economic stress, clinicians in developing countries preferred to choose EGFR-TKIs as the first-line targeted drugs for NSCLC patients with co-mutations of EGFR and ALK genes. Moreover, Yang *et al*^[20] reports for the first time that EGFR-TKIs-treated patients with EGFR/ALK-L1152R mutations generally had a shorter PFS than patients with other mutation combinations (the mPFS of 4 mo vs 18.2 mo, $P < 0.05$).

According to the principle of staging treatment of NSCLC, stage I, II and III resectable NSCLC patients preferred surgical treatment; Simultaneous radical radiotherapy and chemotherapy were the main choice for stage III unresectable NSCLC; stage IV NSCLC patients were treated systemically. First-line targeted therapeutic drugs such as gefitinib, erlotinib, and crizotinib are recommended for advanced NSCLC patients with specific oncogene mutations such as EGFR 19del, L858R, and ALK rearrangement. In our study, Follow-up data collected from six LUAD patients with co-mutations of EGFR and ALK genes showed that four cases selected EGFR-TKIs as first-line treatment, one case as second-line treatment and another case as third-line treatment. Here, we focus on the case 6#. The case 6# was a female patient with left upper LUAD (Figure 3A and B) with right hip and left thalamus metastasis. Genetic testing revealed L861Q mutation and ALK rearrangement by real-time PCR. Starting in June 2018, the patient received 150 mg of erlotinib once daily, and further reduction of the left upper lung lesion was thought to be partial response after targeted therapy (Figure 3C and D). After taking erlotinib for 4 mo, her chest CT presented that multiple lymph nodes in left hilum and mediastinum were significantly larger than before, indicating disease progressed (Figure 3E), then she added crizotinib combined with erlotinib for treatment on her own, further enlargement of left hilar and mediastinal lymph nodes (Figure 3F) after 15 d suggesting poor response to targeted therapy. Later, she was treated with bevacizumab combined with pemetrexed combined with carboplatin for 2 courses, subsequent review of chest CT showed that the left upper lung lesion and left hilar and

mediastinal lymph nodes shrank (Figure 3G and H), suggesting chemotherapy was effective. Fearing the side effects of chemotherapy, she refused further chemotherapy, and sent the mediastinal lymph nodes biopsy sample for NGS, revealing G719A and L861Q mutations. She then took afatinib orally by herself for targeted therapy, but soon died of massive hemoptysis. In our opinion, although the case 6# received multiline treatment including targeted therapies, chemotherapy, and anti-vascular therapy, and its therapeutic strategy had been adjusted several times in time, the rapid progression of the disease itself and the emergence of new EGFR mutation during targeted therapy ultimately led to poor efficacy of targeted therapy. The retrospective analysis of the follow-up data of six LUAD patients with co-mutations of EGFR and ALK genes showed that EGFR-TKIs were their preferred targeted drugs for first-line targeted therapy, and the PFS ranged between two months and six months, suggesting that the therapeutic effect is not good.

Due to tumour heterogeneity, the optimal treatment of co-mutated NSCLC is still challenging. Tumour heterogeneity, including gene mutation abundance and protein phosphorylation heterogeneity, may be associated with the therapeutic effect of TKIs. Zhou *et al*^[21] pointed out that the baseline abundance values of EGFR mutations may be positively correlated with the efficacy of EGFR-TKIs. Yang *et al*^[22] found that EGFR or ALK protein phosphorylation was associated with the efficacy of EGFR-TKIs or ALK-TKIs. Therefore, patients who are relatively sensitive to EGFR-TKIs have higher levels of EGFR protein phosphorylation and lower levels of ALK protein phosphorylation, and vice versa. Lou *et al*^[23] retrospectively analyzed 11 Asian NSCLC patients with co-mutations of EGFR and EML4-ALK genes, and believed that EGFR-TKIs were a feasible option. The subsequent treatment strategy should be further determined according to ALK rearrangement status and EGFR or ALK protein phosphorylation.

It is well known that EGFR gene and ALK gene have a common signaling pathway. ALK rearrangement may lead to EGFR-TKIs resistance in NSCLC patients with EGFR mutation^[23], which is another reason for EGFR-TKIs resistance after KRAS^[25]. On the contrary, EGFR mutation and its signal activation are important molecular mechanisms

of ALK-TKIs resistance^[26,27]. Thus, when tumour cells carry EGFR mutation and ALK rearrangement simultaneously, EGFR-TKIs and ALK-TKIs will eventually acquire drug resistance. Theoretically, EGFR/ALK dual-TKIs may be more effective targeted drugs for NSCLC patients with co-mutations of *EGFR* and *ALK* genes, which needs more real cases to confirm.

Limitations of the study

There are several limitations in this study. First, the cases from a single-Institution enrolled are not large and the collected follow-up data are incomplete, more and more complete cases are needed to further validate our results in multicenter studies in the future. Second, as Shin *et al*^[17] pointed out, since no patient in this study used ALK-TKIs as the first-line therapy, it was not a direct comparison of which targeted drugs are more effective between EGFR-TKIs and ALK-TKIs in NSCLC patients with coexistence of EGFR mutation and ALK rearrangement. Additionally, the specific subtype of ALK rearrangement in this study is unknown, and its effect on TKIs targeted therapy needs to be further evaluated.

CONCLUSION

In Gannan region, the positive rate of LUAD patients with co-mutations of EGFR and ALK genes is relatively high, and the co-mutations are more common in women, non-smoking and stage IV patients with bone metastasis. These patients prefer EGFR-TKIs as their preferred targeted drugs, but the therapeutic effect is not good. EGFR/ALK dual-TKIs may be more effective targeted drugs, which needs further study.

ARTICLE HIGHLIGHTS

Research background

Epidermal growth factor receptor (EGFR) mutation and anaplastic lymphoma kinase (ALK) rearrangement are the most two important therapeutic target, which are significantly associated with the sensitivity of EGFR-tyrosine kinase inhibitors (EGFR-

TKIs) or ALK-TKIs. At present, EGFR-TKIs and ALK-TKIs are widely used in clinical practice and have exhibited favourable anti-tumour effect in the treatment of non-small cell lung cancer (NSCLC). Accumulating evidences confirm that EGFR mutation and ALK rearrangement have coexisted in lung adenocarcinoma (LUAD). However, Its biological mechanism, clinicopathological features, and optimization of targeted drugs have not yet been completely elucidated.

Research motivation

we retrospectively investigated the clinicopathological features and follow-up data of patients with co-mutations of EGFR and ALK genes in LUAD from a single center, aiming to obtain the clinical profile of LUAD patients with co-mutations of EGFR and ALK genes, with intention to scientifically guide the selection of targeted drugs in similar patients, and ultimately achieve individualized precise treatment.

Research objectives

This study aimed to obtain the clinical profile of LUAD patients with co-mutations of EGFR and ALK genes, with hopes of scientifically guiding similar patients towards selected, targeted drugs in similar patients, and ultimately achieve individualized precise treatment.

Research methods

Two hundred and thirty-seven LUAD patients were enrolled. In the experiment, EGFR mutation was detected using amplification refractory mutation system-peptide nucleic acid, and ALK rearrangement was screened by 5'/3' imbalance strategy for reverse transcription followed by quantitative polymerase chain reaction. The clinicopathological features of these patients were analysed retrospectively, and the follow-up data were collected.

Research results

There were six cases with co-mutations of EGFR and ALK genes, hence a positive rate of 2.53% (6/237), and the co-mutations were more common in women, non-smoking and stage IV LUAD patients with bone metastasis. EGFR-TKIs were their preferred drugs for targeted therapy in these patients, with progression-free survival ranging from two months to six months.

Research conclusions

In Gannan region, the positive rate of co-mutations of EGFR and ALK genes in LUAD patients is relatively high, which may be related to regional heterogeneity, and the co-mutations are more common in women, non-smoking and stage IV patients with bone metastasis. These patients prefer EGFR-TKIs as their preferred targeted drugs, but the therapeutic effect is not good. EGFR/ALK dual-TKIs may be more effective targeted drugs.

Research perspectives

In this study, the patients with coexistence of EGFR mutation and ALK rearrangement prefer EGFR-TKIs as their preferred targeted drugs, but the therapeutic effect is not good. Theoretically, EGFR/ALK dual-TKIs may be more effective targeted drugs for NSCLC patients with co-mutations of EGFR and ALK genes, which needs more real cases to confirm. Besides, the cases in this study from a single-Institution enrolled are not large and the collected follow-up data are incomplete, more and more complete cases are needed to further validate our results in multicenter studies in the future. Additionally, the specific subtype of ALK rearrangement in this study is unknown, and its effect on TKIs targeted therapy needs to be further evaluated.

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