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Research Article

# Significance of EGFR Gene Mutation in Fresh Cytological Specimens of Lung Adenocarcinoma

Guo Xiao, Wang Rui, Wu Juan, Ji Xiaokun, Wang Heng, Zhang Yan, Ma Yang, Du Yun

Cancer Detection Center of the Fourth Affiliated Hospital of Hebei Medical University, Shijiazhuang 050011, Hebei, China

**Abstract:** Objective:This paper aims to study the mutation of epidermal growth factor receptor (EGFR) gene in fresh cytological specimens from patients with lung adenocarcinoma, and to determine the prognosis of positive patients by tyrosine kinase inhibitor (TKI). Methods: A total of 313 specimens from needle aspiration and pleural effusion were collected in the Cancer Detection Center of the Fourth Hospital of Hebei Medical University. After HE and immunocytochemistry stainings, the specimens were diagnosed as lung adenocarcinoma by two cytology pathologists. The mutation of 18-21 exon was detectied using ARMS to observe mutations situation. Then, the objective response rate (ORR) and the progression-free survival (PFS) between the targeted group and the chemotherapy group of patients were comparedith. Results: Among 313 cases, 293 cases of lung adenocarcinoma were diagnosed, and DNA specimens were extracted from 288 cases. The success rate was about 98.3%. 130 mutations were found and the rate was 45.1%. EGFR mutation of adenocarcinoma patients mainly occurred to females, nonsmokers, but had nothing to do with age. The ORR was statistically different between the targeted group with chemotherapy ($P$<0.01), and PFS curve of targeted group was on chemotherapy group. The efficacy and the survival time of targeted group and targeted and chemotherapy group were superior to that of chemotherapy group. The results of the EGFR mutation and the prognosis of the tested positive patients in the fresh cytology samples were consistent with that from previous literatures. Conclusion: The results of the test were accurate, and fresh cytological specimens can be used as a replacement for tumor tissue specimens.

**Key words:** epidermal growth factor receptor, fresh cytology specimens, gene mutation, lung adenocarcinoma, targeted therapy, chemotherapy

## 0 Introduction

Epidermal growth factor receptor (EGFR) is a kind of transmembrane glycoprotein with PTK activity. It has relatively high mutation rate in lung adenocarcinoma. In 2005, National Comprehensive Cancer Network of the United States (NCCN) recommended conventional EGFR gene detection in patients with advanced non-small cell lung cancer (NSCLC), and targeted drugs were recommended as first-line therapy for patients with sensitive mutations[1]. At present, most of the specimens detected with EGFR gene mutations are paraffin-embedded specimens of tumor issue[2], which are difficult to obtain from advanced patients. These patients are often accompanied by body surface lymph node metastasis and pleural effusion, so cytological specimens are relatively easy to obtain and adequate in quantity. In this study, fresh cytology specimens were obtained by fine needle aspiration and pleural effusion extraction. EGFR gene mutations were detected after HE staining and specific immunocytochemical diagnosis. Patients with positive EGFR gene mutations took tyrosine kinase inhibitors (TKI) or received chemotherapy. Then the prognoses were evaluated. This paper explores the feasibility and clinical significance of detecting EGFR gene mutations using fresh cytological specimens.

## 1 Materials and Methods

### 1.1 Clinical data

We collected the fresh cytological specimens and the case data of 313 patients with advanced lung adenocarcinoma who had received EGFR gene mutation detection from January 2013 to January

2015 in the Cancer Center of the Fourth Hospital of Hebei Medical University, including 161 male patients and 152 female patients with their total age 2889 and their average age 60, among whom, 169 patients were beyond 60 and 144 patients were under 60. There were 112 fine needle aspiration specimens and 201 pleural effusion specimens. Inclusion criteria for prognosis evaluations included: fresh cytological specimens detected with positive EGFR, no exon 20 mutation, complete case data, no extrapulmonary primary tumors, clearly identifiable and measurable lesions according to response evaluation criteria in solid tumors (RECIST), and one-month survival time after taking first-line drugs.

### 1.2 Specimen collection and treatment

#### 1.2.1 Collection and treatment of needle aspiration specimens

Finevacuum needles were inserted into the lymph node to extract a small amount of contents. One part of the contents used for EGFR gene mutation detection was injected into a 1.5-ml EP tube and stored in a refrigerator at 20℃. The remaining part was used to make 10 cell smears, among which, 2 with ordinary slides were used for conventional diagnosis and 8 with anti-peeling slides were used for immunocytochemical diagnosis. All the cell smears were fixed in 95% ethanol.

#### 1.2.2 Collection and treatment of pleural effusion specimens

The pleural effusions collected were divided into two parts: one part of the pleural effusions (approximately 100-200ml) was poured into a 20-ml centrifuge tube and centrifuged for 2 minutes with 1800 r/min. The supernatant was discarded, and the sediment at bottom was again poured into the centrifuge tube to be centrifuged until all these pleural effusions were completely centrifuged. After the final centrifugation, 5ml of the supernatant used for EGFR gene mutation detection was mixed and placed in a refrigerator at -20℃. The enriched cells in the remaining part of the pleural effusions were collected using a disposable diseased cell collector. The membrane carrying the cells was evenly coated on 10 slides, and their usage and fixation were the same as the above.

The above specimens were stained by HE to find cancer cells. Immunocytochemical staining was used when the cancer cells accounted for 25% of the total cells on the entire slide.

### 1.3 Immunocytochemistry

According toclinical data and other imaging findings, antibodies such as NapsinA, TTF-1, CEA, CK7, CK5/6, P63, P40, Syn, CD56, E-cadherin, and CR were selected and SP method was used for immunocytochemical staining. The anti-peeling slides were removed from the stationary liquid, washed with water after air drying, then placed in hydrogen peroxide for 15 minutes. After full washing, antigen retrieval was applied. Then we dropped animal non-immune serum to the slides and incubated them at room temperature for 15 minutes. After discarding the excess serum, primary antibodies were added. Then the slides were left staying overnight at 4℃. After PBS washing, secondary antibodies were added. After a 20-minute incubation, another PBS washing and DAB staining, we used hematoxylin to restain the cell nucleus, and then applied alcohol differentiation, ammonium hydroxide, and gradient alcohol dehydration. Finally, the slides was blown dry and sealed with neutral gum.

After immunocytochemical labeling, the target cells were observed. Only when the location of antibody staining was accurate and pale brown or brown particles were found at corresponding sites can the specimen be evaluated as positive.

### 1.4 EGFR gene mutation detection

#### 1.4.1 DNA extraction

The QIAamp DNA Mini Kit of German Company QIAGEN was applied. After digestion, combination, washing and elution, the purity and concentration of the DNA samples were detected under ultraviolet spectrophotometer to ensure the purity of 1.7-2.1 and the concentration of 2-10ng/μL.

#### 1.4.2 EGFR gene mutation detection

The human EGFR gene mutation detection kit developed by Wuhan Hygeianey Biotechnology Co., Ltd. was used for detection. This kit used amplification refractory mutation system (ARMS) technology for real-time fluorescence quantitative PCR to detect mutations in exon 1821 of the human EGFR gene. The main steps are, first, respectively adding 4μL of the hot-start Taq DNA polymerase to 40μL of the DNA sample, 40μL of the negative control and 40μL of the positive control, then, after the instantaneous centrifugation, respectively putting 5μL of each sample to the 8-hole

reaction strip of the kit, placing it in the ABI7500 real-time fluorescence quantitative PCR, and setting up the program following the instructions.

### 1.4.3 Test result interpretation

Only when the results collected by PCR thermocycle instrument meet the following conditions can they be regarded as credible. ① The negative control rises no FAM signal. ② The $C_T$ value of the FAM signal of positive control is between 10 and 20. ③ The $C_T$ value of the FAM signal of the external control reaction hole (the 8th hole of the EGFR 8-hole reaction strip) is between 10 and 25.

This kit usesthe value of $\triangle C_T$ to interpret the results. After completion, each sample has 7 $C_T$ value of the mutation signal corresponding to detection system (FAM), 7 $C_T$ value of the internal control signal(JOE/VIC), and 1 $C_T$ value of external control signal (FAM). If the $C_T$ value of the mutation signal is greater than 30, the test result is negative. If the $C_T$ value of the mutation signal is less than or equal to 30, the value of $\triangle C_T$ between the detection system and the FAM signal of the external control detection system should be calculated. Mutations in the EGFR gene are distributed in exon 18-21, so there may be multiple mutations coexisting (see Table 1).

The value of$\triangle C_T$=The $C_T$ value of the mutation signal (FAM)-The $C_T$ value of the external control signal(FAM)

**Table 1 EGFR Mutation Content Determination**

| Detection System | G719X | 19del | T790M | S768I | 20ins | L858R | L861Q |
|---|---|---|---|---|---|---|---|
| Positive $\Delta C_T$ | <8 | <9 | <10 | <8 | <7 | <9 | <8 |
| Mutation Content | ≥ 1% | ≥ 1% | ≥ 1% | ≥ 1% | ≥ 1% | ≥ 1% | ≥ 1% |
| Negative $\Delta C_T$ | ≥ 8 | ≥ 9 | ≥ 10 | ≥ 8 | ≥ 7 | ≥ 9 | ≥ 8 |
| Mutation Content | <1% | <1% | <1% | <1% | <1% | <1% | <1% |

G719X is exon 18 mutation. 19del is exon 19 mutation. T790M, S768I, and 20ins are exon 20 mutations. L858R and L861Q are exon 21 mutations.

### 1.5 Prognosis evaluation

Patients with positive EGFR gene mutations were screened according to the criteria listed in 1.1 and divided into two groups: targeted therapy group and chemotherapy group based on their first-line medication situations. Targeted therapy group took orally 150mg/d of Erlotinib or 250mg/d of Gefitinib. Chemotherapy group were applied intravenous drip of 30mg/m$^2$ of Cisplatin on the first three days, and 135 mg/m$^2$ of Paclitaxel on the first day. Every three weeks is a course of treatment. All enrolled patients were continuously treated until the treatment made some progress or intolerable side effects appeared. During the treatment, the patients were followed up every 3 months to observe their objective response rate (ORR) and progression free survival (PFS).

### 1.6 Statistical analysis

SPSS 16.0 statistical analysis software was used tocalculate $X^2$ for the test and evaluation of ORR. Kaplan-Meier curve was used for PFS analysis.

## 2 Results

### 2.1 The results of HE staining and immunocytochemical staining

Among313 patients, cancer cells were found in 297 patients, among whom, 293 were diagnosed with lung adenocarcinoma.

### 2.2 EGFR gene mutation test results

Among the 293 patients with lung adenocarcinoma, DNA was successfully extracted from 288 patients. The success rate of extraction was about 98.3%. These 288 patients were tested for EGFR gene mutations, and gene mutations were found in 130 patients. The mutation rate was 45.1%. Among them, there were 5 cases of exon 18 mutation, accounting for 3.8%, 60 cases of exon 19 mutation, accounting for 46.2%, 5 cases of exon 20 mutation, accounting for 3.8%, and 60 cases of exon 21 mutation, accounting for 60%. The relationship between EGFR mutation and the clinical features of patients is shown in Table 2.

**Table 2 The relationship between EGFR mutation and clinical features**

| Clinical Features | Overall Number of Patients | TheNumber of Patients with Mutation | Mutation Rate | *P* | |
|---|---|---|---|---|---|
| Gender | Male | 148 | 55 | 37.20% | 0.005 |
| | Female | 140 | 75 | 53.60% | |
| Age | ≥ 60 | 155 | 71 | 45.80% | 0.806 |
| | <60 | 133 | 59 | 44.40% | |
| Whether They Smoke | No | 195 | 97 | 49.70% | 0.023 |
| | Yes | 93 | 33 | 35.50% | |

### 2.3 Prognosis

By January 2016, among the 93 patients with positive EGFR who met the inclusion criteria, 54 patients had died. The median follow-up time was 14 months. The difference in ORR between targeted therapy group and chemotherapy group is statistically significant ($X^2 \approx 24.544$, $P<0.01$), which means the efficacy of targeted therapy group was superior to that of chemotherapy group (see Table 3). The median PFS of targeted therapy group and chemotherapy group was 9.5 months and 6 months, respectively. It can be seen from Figure 1 that the curve of targeted therapy group is above the curve of chemotherapy group, which means the PFS of targeted therapy group is longer than that of chemotherapy group.

**Table 3 Comparison of the curative effects of 3 groups of patients with NSCLC**

| Group | Total Number of Cases | CR | PR | SD | PD | ORR(%) | $\chi^2$ | *P* |
|---|---|---|---|---|---|---|---|---|
| Targeted Therapy Group | 44 | 3 | 33 | 5 | 3 | 81.8 | 24.544 | <0.01 |
| Chemotherapy Group | 49 | 0 | 15 | 13 | 21 | 30.6 | | |

CompleteRemission (CR), Partial Remission (PR), Stable Disease (SD), Progression Disease(PD). The objective response rate (ORR)=(CR+PR)/ total number of cases. The disease control rate (DCR)=(CR+PR+SD)/ the total number of cases.

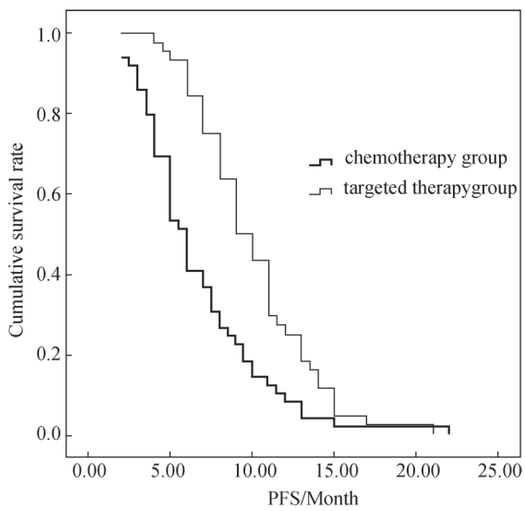


**Figure 1**

## 3 Conclusion

Today,as a serious threat to human health, lung cancer is one of the most malignant tumors with the highest morbidity and mortality. Non-small cell lung cancer is the most common type of lung cancer[3]. Due to its occult onset, most of the patients were diagnosed with advanced cancers at first visit. Therefore, they lost the operation chance, and their prognoses were poor. Although platinum-based chemotherapy is the standard medication for advanced non-small cell lung cancer, it can cause toxic and side effects such as myelosuppression, liver and kidney injury, thus reducing the quality of patients'lives[4]. With the development of oncomolecularbiology, targeted therapy has become more and more important due to its high selectivity, small side effects and good curative effects[5-7]. However, targeted drugs are expensive and they cannot benefit all patients. Only by selecting suitable targets can it provide basis for personalized treatment of lung cancer. In China, EGFR gene mutations are mostly found in non-small cell lung cancers[8]. The study of this targeted spot has become a hot topic recently. At present, most of the EGFR gene mutation detection is to remove the paraffin-embedded specimens of tumor issue through operations, which is difficult for patients with advanced lung cancer who have lost the operation chance. Therefore, the highest priority is to find an alternative specimen for EGFR gene mutation detection.

AsEGFR gene mutation often occurs in women, non-smokers, and patients with lung adenocarcinoma[9], the nature and sources of body surface lymph node enlargement and pleural effusion should be clarified first. Body surface lymph node enlargement is more common in patients with advanced lung cancer. It is difficult to determine the nature of the enlarged lymph nodes only by its location, appearance, and imaging test. Its primary site can be everywhere of the body. Pleural effusion is also common in lung diseases, especially in lung adenocarcinoma. However, conventional cytological examination mainly depends on the morphological characteristics of cancer cells, but cancer cells lost their original morphology in effusion. In addition, the morphology of mesothelial cells has some morphological features of "cancer cells" after the reactive hyperplasia when stimulated. Therefore, it's difficult to make judgments based on morphology alone. It requires immunocytochemistry to assist in diagnosis. At the same time, there may be many non-tumor cells in fresh cytological specimens, so a method with high sensitivity is needed. The amount of tumor tissue

required by ARMS method is small. DNA sample with 1% of mutation content under the background of 10ng genome[10] is enough to test the situation of EGFR mutation. Therefore, obtaining fresh cytological specimens through fine needle aspiration and pleural effusion extraction is easy to operate. It has advantages of small wound, low cost, good repeatability, and adequate specimen sources. The specimens are also easy to collect. To some extent, it can solve the problem of specimen collection.

Among the288 fresh cytological specimens of the study, the total mutation rate of exon 1821 was 45.1% (130/288). It's basically consistent with the mutation rate of EGFR gene reported in reference [11], which is 36.4%-66.3%. According to the reports of Kasaka et al.[12], Paez et al.[13], and Lynch et al. [14], 90% of EGFR mutations occur in exon 19 and exon 20. In this study, exon 19 mutation and exon 21 mutation occurred in 120 patients, and the total mutation rate of the two kinds of mutations accounted for 92.4% (120/288), which is also consistent with the reports. At the same time, we know the objective response rate and progression free survival of the the patients with positive EGFR in targeted therapy group are superior to those of the patients in chemotherapy group, which is the same as the results of IPASS study[15], NEJGSG002 study[16], WJTOG 3405 study[17], and OPTIMAL study[18]. Therefore, it can be concluded that the results are accurate using fresh cytological specimens for EGFR detection.

Inconclusion, when tumor cell specimens can't be clinically obtained, it's effective to use fresh cytological specimens as alternative specimens.

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