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Original Article

Serum EGFR gene mutation status via second-generation sequencing and clinical features of patients with advanced lung cancer

Zhen Zhang[1], Shengyu Zhou[2]

[1]*Department of Lung Cancer, Tianjin Medical University Cancer Institute and Hospital, Tianjin's Clinical Research Center for Cancer, Tianjin's Key Laboratory of Cancer Prevention and Therapy, National Clinical Research Center for Cancer, Tianjin, China;* [2]*Department of Medical Oncology, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China*

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Abstract: *Objective:* To detect the serum epithelial growth factor receptor (EGFR) gene mutation status in patients with lung cancer via second-generation sequencing and to analyze its correlations with the clinical features of patients and its therapeutic effects. *Methods:* A total of 110 patients with non-small cell lung cancer (NSCLC) treated in our hospital were recruited as subjects of our study. The distribution of the EGFR gene mutation in patients was detected via second-generation sequencing and then patients were divided into mutant-type EGFR group (n=37) and wild-type EGFR group (n=73). The clinical features and therapeutic effects were compared between the two groups of patients. *Results:* A total of 5 kinds of EGFR gene mutation [19del (45.95%), L858R (43.24%), L861Q (5.41%), S768I (2.70%), and G719X (2.70%)] were detected via second-generation sequencing. In the mutant-type EGFR group, the proportions of female patients, patients with adenocarcinoma, and those with no history of smoking were high, and the differences were statistically significant (P<0.05). Moreover, there were statistically significant differences in gender, type of cancer, tumor-node-metastasis (TNM) staging, and smoking history between the mutant-type EGFR group and the wild-type EGFR group (P<0.05). Results of a multivariate logistic regression analysis showed that the EGFR gene mutation status was significantly associated with the type of cancer, gender, TNM staging, and smoking history of patients with NSCLC (P<0.05). The clinical effective rate of patients in the mutant-type EGFR group was significantly higher than that in the wild-type EGFR group (54.05% vs. 19.18%) while progression-free survival (PFS) was significantly longer than that in the wild-type EGFR group [(9.75±1.64) months vs. (5.51±0.40) months] (P<0.05). The expression level of carcinoembryonic antigen (CEA) in patients in the mutant-type EGFR group was apparently higher than that in the wild-type EGFR group, but the levels of carbohydrate antigen 125 (CA125), CY21-1 and squamous cell carcinoma-related antigen (SCC-Ag) were apparently lower than those in the wild-type EGFR group. There were statistically significant differences in the expression levels of serum tumor markers between the two groups (P<0.05). *Conclusion:* EGFR mutation status is closely related to the clinical features of NSCLC patients, such as gender, type of cancer, tumor staging, smoking history, and clinical effect. The second-generation sequencing is an important detection method to identify EGFR gene mutation status, which provides an applicable scaffold for the clinical treatment of patients.

Keywords: Second-generation sequencing, lung cancer, epidermal growth factor receptor gene, gene mutation, clinical features

Introduction

The epidermal growth factor receptor (EGFR) gene is a common mutant gene in patients with advanced non-small cell lung cancer (NSCLC). The first-line drug for the treatment of lung cancer patients with EGFR gene mutation represents EGFR tyrosine kinase inhibitors (EGFR-TKIs) [1-3]. Statistical results show that EGFR gene mutation detection is performed among only a small number of lung cancer patients in China before the therapy method is chosen. One of the major reasons is the difficulty of tumor tissues collection. Therefore, detecting

Table 1. Primer sequences

Exon	Forward primer	Reverse primer
Exon 18	5'-GAGGTGACCCTTGTCTCTGTGT-3'	5'-CCCAAACACTCAGTGAAACAAA-3'
Exon 19	5'-TGCCAGTTAACGTCTTCCTTCT-3'	5'-TGAACATTTAGGATGTGGAGAT-3'
Exon 20	5'-ACTTCACAGCCCTGCGTAAAC-3'	5'-ATGGGACAGGCACTGATTTGT-3'
Exon 21	5'-GAGCTTCTTCCCATGATGATCT-3'	5'-GAAAATGCTGGCTGACCTAAAG-3'

EGFR gene mutation status without the extraction of tumor tissues of patients can effectively improve the screening of the EGFR gene status of lung cancer patients in China, which is of great importance for the development of the therapeutic regimen. At present, researchers have confirmed that the tumor deoxyribonucleic acid (DNA) level in the peripheral blood of patients with lung cancer is generally higher than that in healthy people. Moreover, a large number of researchers compared the EGFR levels in serum and tumor tissue samples and found that the consistency between the two was as high as 60%-95% [4-6]. Direct sequencing, the "gold standard" for detecting gene mutation status, has the advantages of high accuracy and strong specificity. In recent years, the second-generation sequencing emerged and has been gradually applied in tumor susceptibility and personalized medication. In this study, 110 patients with NSCLC treated in our hospital were recruited for this study. The distribution of the serum EGFR gene mutation in NSCLC patients was detected via second-generation sequencing and the correlations among the EGFR gene mutation status the patients' clinical features, and the therapeutic effect of EGFR-TKIs were further analyzed.

Materials and methods

General materials and grouping

In this study, 110 patients with NSCLC treated in our hospital from December 2016 to June 2017 were selected as objects of study. According to results of the second-generation sequencing, they were divided into patients with mutant-type EGFR (mutant-type EGFR group, n=37) and patients with wild-type EGFR (wild-type EGFR group, n=73). The study was reviewed and approved by the Ethics Committee of our hospital and all patients who participated in the study signed the informed consent.

Inclusion criteria

1) Patients with advanced NSCLC confirmed via cytology or histopathology, and 20-65 years old in either gender, 2) Patients with the general performance status score of 0-3 points, 3) Patients with at least one measurable lesion, 4) Patients who did not receive any drug therapy and radiotherapy before enrollment, and 5) Patients with complete clinical data.

Exclusion criteria

1) Patients complicated with major organ diseases, 2) Patients with abnormal results in three major routine examinations: blood, urine, and feces, 3) Patients with hepatic and renal dysfunction or severe cardiac dysfunction, 4) Patients complicated with other malignant tumors, or 5) Patients with incomplete clinical data.

Sample collection and DNA extraction

Before treatment, 5 mL of peripheral venous blood was collected from all subjects, placed at room temperature for 2 h and centrifuged at 3,000 rpm in a low-temperature, high-speed centrifuge for 10 min. Then the upper-layer plasma was taken, placed into a 2 mL cryogenic tube, and stored in a freezer at -80°C to be tested. DNA was extracted from the plasma using the blood DNeasy kit (Qiagen, Hilden, Germany) in strict accordance with the kit's instructions. The concentration and purity of DNA were detected using a ultramicro spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA), and the optical density $(OD)_{260}/OD_{280}$ ratio of the DNA sample had to be between 1.8 and 2.0.

Detection of EGFR gene mutation status via second-generation sequencing

The EGFR gene sequence was based on the sequence (No. AY588246) published by GenBank, and the primer was designed using ABI Prism™ Primer Express software (Waltham, MA, USA). Primer sequences are shown in **Table 1**.

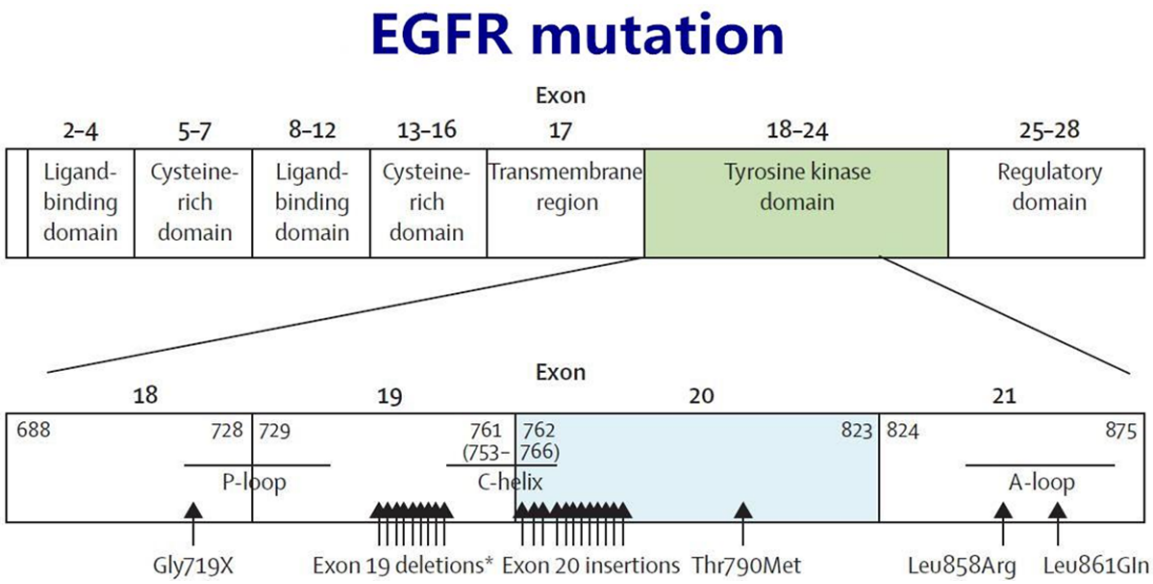

Figure 1. EGFR mutation.

Table 2. Distribution of the EGFR gene mutation in patients with advanced lung cancer ($\bar{x}$ ±s, n=37)

Mutation	Case (n)	Ratio (%)
19del	17	45.95
L858R	16	43.24
L861Q	2	5.41
S768I	1	2.70
G719X	1	2.70

The exons 18, 19, 20, and 21 of the EGFR gene were amplified via polymerase chain reaction (PCR). PCR amplification products were purified, followed by bi-directional sequencing using the ABI Ion Proton™ second-generation sequencer, and the analysis of the sequencing results was done using Chromas software (Waltham, MA, USA). Reverse sequencing was performed using reverse primers to verify mutant or deleted samples (**Figure 1**).

Observation indexes

1) Second-generation sequencing was used to analyze the distribution of EGFR gene mutations in patients with advanced NSCLC. 2) The clinical features, such as gender, age, type of cancer, tumor-node-metastasis (TNM) staging, and smoking history, were recorded and compared between the two groups of patients. 3) Multivariate logistic regression analysis was used to analyze the correlations of serum EGFR gene mutation status with the clinicopathological features of patients with NSCLC. 4) The therapeutic effects of the two groups of patients were evaluated, and the evaluation criteria of the curative effects were as follows: complete response (CR): all target lesions disappeared, there were no new lesions, and tumor markers reached the normal level for more than 4 weeks. Partial remission (PR): The sum of the maximum diameters of all target lesions was increased by less than 20% or decreased by less than 30%. Progressive disease (PD): New lesions appeared, or the sum of the maximum diameters of all original target lesions was increased by more than 20%. Response rate (RR)=(CR+PR)/total number of patients ×100%. 5) The median progression-free survival (PFS) was compared between the two groups. PFS was from the start of treatment for patients to the last follow-up of disease progression or no progression. The deadline for the follow-up was December 31, 2017. 6) Expression levels of serum tumor markers, such as carcinoembryonic antigen 125 (CA125), squamous cell carcinoma-related antigen (SCC-Ag), CA19-9 and cytokeratin 19 fragment (CYFRA21-1), in both groups of patients were detected via the electrochemiluminescence method using the full-automatic electrochemiluminescence immunoassay analyzer (Roche, Indianapolis, IN, USA).

Table 3. Comparisons of general data and clinical features between the two groups of subjects [n (%)]

Clinical feature		Case (n)	Mutant-type EGFR group (n=37)	Wild-type EGFR group (n=73)	χ^2	*P*
Gender	Male	63	15/63 (41.67)	48/63 (58.33)	4.526	<0.05
	Female	47	22/47 (46.81)	24/47 (53.19)		
Age (years old)	<65	60	24/60 (40.00)	36/60 (60.00)	1.286	>0.05
	≥65	50	13/50 (26.00)	37/50 (74.00)		
Type of cancer	Adenocarcinoma	80	33/80 (41.25)	47/80 (58.75)	5.487	<0.05
	Non-adenocarcinoma	30	4/30 (13.33)	26/30 (86.67)		
TNM staging	Stage IIIb	21	5/21 (23.81)	16/21 (76.19)	0.428	>0.05
	Stage IV	89	32/89 (35.96)	57/89 (64.04)		
Smoking history	Yes	66	17/66 (25.76)	49/66 (74.24)	4.025	<0.05
	No	44	20/44 (45.45)	24/44 (54.55)		

Table 4. Multivariate logistic analyses of EGFR gene mutation status and clinicopathological features

Factor	Regression coefficient (β)	Standard error (SE)	Wald value	*P*	Odds ratio (OR) [95% confidence interval (CI)]
Age	0.368	0.349	1.118	>0.05	0.771 (0.684-1.630)
Type of cancer	2.019	0.684	7.265	<0.05	5.260 (1.123-7.067)
Gender	2.035	0.525	8.291	<0.05	8.049 (4.259-12.184)
TNM staging	0.410	0.426	1.076	>0.05	0.720 (0.636-2.851)
Smoking history	1.359	0.551	4.217	<0.05	3.336 (1.337-6.561)

Statistical processing

In this study, all data were processed using Statistical Product and Service Solutions (SPSS) 20.0 statistical analysis software (IBM, Armonk, NY, USA). Measurement data were presented as mean ± standard deviation, and a one-way analysis of variance or repeated measures analysis of variance with Tukey's post hoc test was used for the comparison among the groups. The least significant difference (LSD)-*t* test was used for the pairwise comparison. Enumeration data were presented as a percentage (%), and a chi-square analysis was used for the comparison among the groups. *P*<0.05 suggested that the difference was statistically significant.

Results

Distribution of EGFR gene mutation in patients with advanced lung cancer

In 37 patients with EGFR gene mutation, a total of 5 kinds of mutation [19del (45.95%), L858R (43.24%), L861Q (5.41%), S768I (2.70%) and G719X (2.70%)] were detected (**Table 2**).

Comparisons of clinical features between the two groups

In the mutant-type EGFR group, the proportion of female patients was significantly higher than that of male patients (*P*<0.05). The proportion of patients with adenocarcinoma was significantly higher than that of non-adenocarcinoma patients (*P*<0.05). The proportion of patients in stage IIIb was significantly lower than that of patients in stage IV, and the proportion of patients with a smoking history was statistically lower. The differences were statistically significant (*P*<0.05). There were also statistically significant differences in gender, type of cancer, TNM staging, and smoking history between the mutant-type EGFR group and the wild-type EGFR group (*P*<0.05) (**Table 3**).

Multivariate logistic analyses of EGFR gene mutation status and clinicopathological features

Results of a multivariate logistic regression analysis showed that the EGFR gene mutation status was significantly associated with the type of cancer, gender, TNM staging and the smoking history of patients with NSCLC (*P*<

Table 5. Evaluation of short-term efficacy in the two groups [n (%)]

Group	CR	PR	SD	PD	Clinical effective rate
Mutant-type EGFR group (n=37)	1/37 (2.70)	19/37 (51.35)	14/37 (37.84)	3/37 (8.11)	20/37 (54.05)
Wild-type EGFR group (n=73)	0/73 (0.00)	14/73 (19.18)	49/73 (67.12)	9/73 (12.33)	14/73 (19.18)
χ^2	-	-	-	-	5.873
p	-	-	-	-	<0.05

Table 6. Comparison of survival time after treatment between the two groups ($\bar{x}\pm s$)

Group	Case (n)	PFS (months)
Mutant-type EGFR group	37	9.75±1.64
Wild-type EGFR group	73	5.51±0.40
t	-	15.494
p	-	<0.05

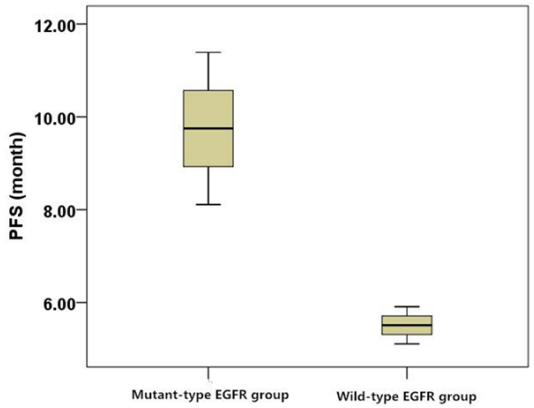

Figure 2. Survival time of patients after treatment.

0.05), but it was not significantly associated with age (**Table 4**).

Evaluation of short-term efficacy in two groups of subjects after treatment

After treatment, there was 1 case of CR, 19 cases of PR, 14 cases of SD, and 3 cases of PD, respectively, in the mutant-type EGFR group. Additionally, there were 0 cases of CR, 14 cases of PR, 49 cases of SD, and 9 cases of PD, respectively, in the wild-type EGFR group. The clinical effective rate of patients in the mutant-type EGFR group was significantly higher than that in the wild-type EGFR group (54.05% vs. 19.18%) (*P*<0.05) (**Table 5**).

Comparison of survival time after treatment between the two groups of subjects

Follow-up results showed that PFS in the mutant-type EGFR group [(9.75±1.64) months] was significantly longer than that in the wild-type EGFR group [(5.51±0.40) months] (*P*< 0.05) (**Table 6**; **Figure 2**).

Comparisons of expression levels of serum tumor markers between the two groups of subjects

The expression level of CEA in subjects in the mutant-type EGFR group was higher than that in the wild-type EGFR group, but the levels of CA125, CY21-1 and SCC-Ag were lower than those in the wild-type EGFR group, and there were statistically significant differences in the expression levels of serum tumor markers between the two groups (*P*<0.05) (**Table 7**; **Figure 3**).

Discussion

According to statistics, both the incidence and fatality rates of lung cancer rank first among malignant tumors in China and even in the world. NSCLC accounts for about 85% of lung cancer cases, which seriously threatens people's health and life [7]. Targeted drugs represented by EGFR-TKIs have a significant therapeutic effect on NSCLC patients with the EGFR gene mutation, whereas patients with wild-type EGFR will not benefit from EGFR-TKIs drug therapy [8, 9]. Therefore, in order to improve the therapeutic effect, the EGFR gene mutation status should be cleared before starting the clinical treatment of NSCLC patients [10]. The preferred material for EGFR gene mutation detection extracting a specimen from patients' tumor tissue. However, as tumor tissue specimens of advanced NSCLC patients are mainly bronchoscopic biopsies or lung puncture specimens, the sample sizes are too small for genetic detection. In addition, tumor heterogeneity may reduce the accuracy of tumor biopsy specimens in reflecting the overall gene mutation of tumors. In recent years, researchers have proposed that blood samples be used as biomaterials for genetic detection. A large number of studies have confirmed that EGFR mutation

Table 7. Comparisons of the expression levels of serum tumor markers between the two groups ($\bar{x} \pm s$)

Group	CEA (ng/mL)	CA125 (U/mL)	CY21-1 (ng/mL)	SCC-Ag (ng/mL)
Mutant-type EGFR group (n=37)	25.71±4.82	33.26±5.03	3.28±0.57	0.83±0.19
Wild-type EGFR group (n=73)	11.02±2.02	67.12±9.86	5.87±1.14	1.24±0.22
t	17.765	23.850	15.885	9.653
p	<0.05	<0.05	<0.05	<0.05

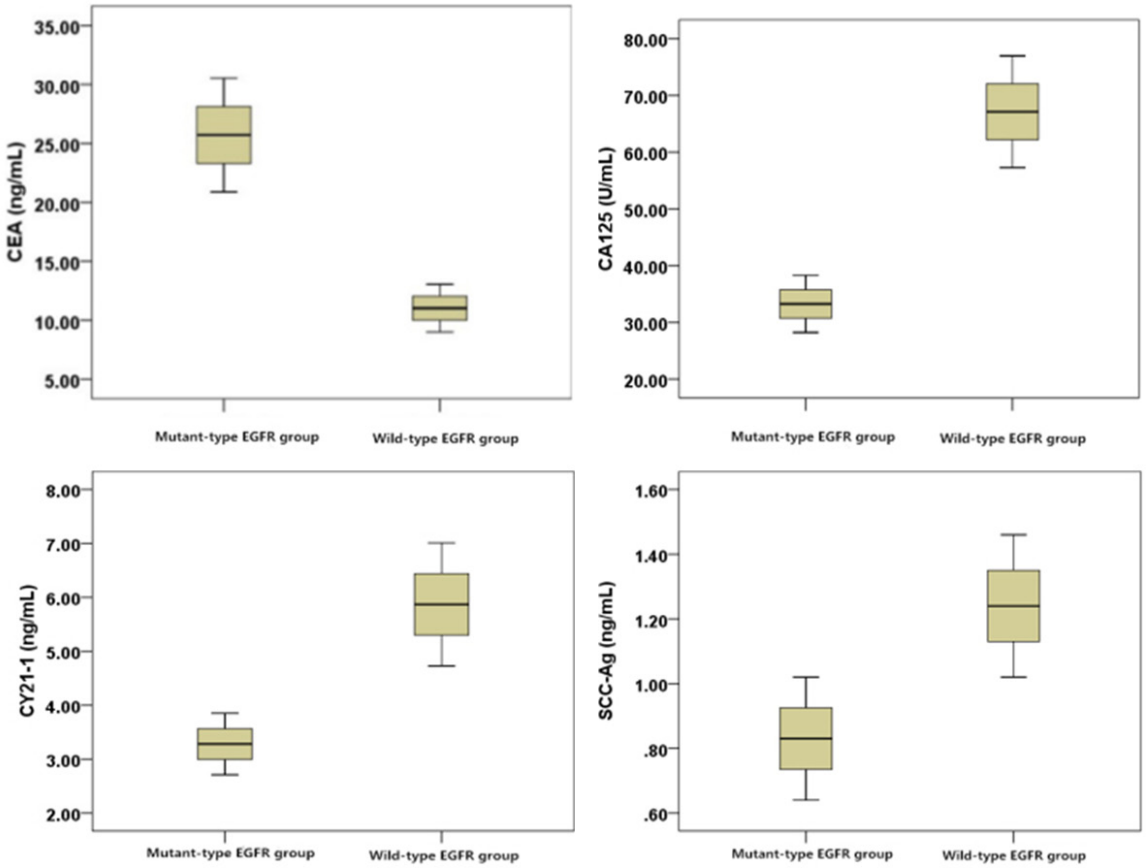

Figure 3. Expression levels of serum tumor markers.

detection results in peripheral blood and tissue specimens of patients with NSCLC are highly consistent, suggesting that second-generation sequencing presents a high feasibility in detecting the serum EGFR gene mutation status of NSCLC patients [11-13].

In this study, the second-generation sequencing technique was adopted to detect the serum EGFR gene mutation status in 110 patients with advanced NSCLC treated in our hospital, and it was found that patients with the EGFR gene mutation accounted for about 33.64% of the patients (n=37), which was consistent with the conclusion of the same type of study [14, 15]. In patients with the EGFR gene mutation, a total of 5 kinds of mutation sites [19del (45.95%), L858R (43.24%), L861Q (5.41%), S768I (2.70%). and G719X (2.70%)] were found. The study on clinical features revealed that, in patients with the EGFR gene mutation, the proportion of females was higher than that of males, the proportion of patients with adenocarcinoma was higher than that of the non-adenocarcinoma patients, the proportion of patients with a smoking history was higher than that of patients without a smoking history, and the proportion of patients in stage IIIb was lower than that of patients in stage IV. Furthermore, results of a multivariate logistic

regression analysis showed that the EGFR gene mutation status was significantly associated with the type of cancer, gender, TNM staging and smoking history of patients with NSCLC. Growing evidence has indicated that the EGFR gene is the first-generation driver gene of NSCLC patients, and its mutation rate is higher in Asian, female, adenocarcinoma and non-smoking patients [16, 17], which is consistent with the results of this study.

Previous findings pointed out that the clinical effective rate and disease control rate were 40% and 70% in first-line therapy using EGFR-TKIs for advanced NSCLC patients with the EGFR gene mutation, and the average PFS of patients was more than 9 months. Another study confirmed that after treatment with gefitinib, the clinical effect and PFS of NSCLC patients with the serum EGFR gene mutation were significantly superior to those after treatment with a standard chemotherapy regimen. Moreover, researchers found that, through the comparative analysis of free DNA in tissues and plasma of patients with advanced lung cancer, both of them were highly consistent in predicting the clinical therapeutic effect of targeted drugs [18-20]. In this study, it was found that the clinical effective rate and PFS of patients in the mutant-type EGFR group were significantly higher than those in the wild-type EGFR group, which were consistent with conclusions of the same type of study. Besides, it was also found in this study that expression levels of serum tumor markers were significantly different between patients with mutant-type EGFR and wild-type EGFR, and its mechanism needs further analysis. However, the limitation of second-generation sequencing still exists and it is noteworthy that the detection of serum EGFR gene mutation status via the second-generation sequencing is not applicable to all patients with lung cancer, because the level of tumor DNA in the blood is affected by tumor staging. The serum EGFR detection rate is frequently higher in patients in stages III and IV, while the detection sensitivity is lower in patients in stages I and II, which is also the main reason why patients with advanced NSCLC were selected for this study.

Conclusion

EGFR mutation status is closely related to the clinical features of NSCLC patients (such as gender, type of cancer, tumor staging, and smoking history), clinical effect and expression levels of tumor markers. The second-generation sequencing presents as a valuable detection method of EGFR gene mutation status, which shows guiding significance in the clinical treatment of patients.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shengyu Zhou, Department of Medical Oncology, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 17 Pan Jia Yuan Nan Li, Chao Yang District, Beijing 100021, China. Tel: +86-8610-87788293; Fax: +86-8610-87788293; E-mail: shengyuzhou123@163.com

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