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Original Article

Identification of EGFR mutations in cytological specimens of non-small cell lung carcinoma from a single institute

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Abstract: Epidermal growth factor receptor (EGFR) mutation testing is pivotal for the precise treatment of non-small cell lung carcinoma (NSCLC). Generally, detection of EGFR mutations in surgical specimens is the most accurate and the most reliable. However, some NSCLC patients in advanced stages are inoperable. Therefore, other specimens such as cytological samples are alternative options for EGFR mutation detection. In this study, cytological samples from 291 NSCLC patients were tested for EGFR mutations using the amplification-refractory mutation system (ARMS). The results showed that the EGFR mutation rate was 45.3%, and the most common mutation type was deletion in exon 19 (19del), followed by L858R mutation in exon 21. The results were consistent with that of the large cohort histological samples. This study demonstrates that using cell block for EGFR mutation detection has a high success rate and can yield reliable results. It also solidifies that the use of cytological samples possesses relatively high clinical practicability, so that cytological samples may substitute histological samples. In addition, an assessment of the tumor cell quantity prior to detection could improve the positive rate. Considering tumor heterogeneity, both histological and cytological samples are suggested to be collected for testing, if possible.

Keywords: EGFR, NSCLC, cytological samples, mutation test, ARMS

Introduction

Lung cancer is the leading cause of cancer-related deaths, both worldwide and in China. Non-small cell lung carcinomas (NSCLCs) comprise approximately 85% of all lung carcinomas [1]. NSCLCs are so insidious that most patients are detected at the advanced stage, and surgical treatment is not recommended. For some of these patients, diagnoses of NSCLCs are based on cytological specimens, such as sputum, pleural fluid, bronchial brushing, or fine-needle aspiration (FNA) specimens [2]. The epidermal growth factor receptor (EGFR) is the most common driver gene that leads to the development of NSCLC. EGFR mutations occur in 30% to 50% of Asians with NSCLCs [3]. The vast majority of EGFR mutations are the deletion in exon 19 and a point mutation in exon 21, which results in a leucine to arginine substitution at codon 858 (L858R) [4]. Targeted tyrosine kinase inhibitors (TKIs), such as erlotinib and gefitinib, which came into use ten years ago, can improve progression-free survival (PFS) in NSCLCs patients that harbor these mutations [5]. Therefore, molecular testing for NSCLCs cannot be emphasized enough. For these patients which were diagnosed as NSCLCs based on cytological specimens, the molecular test can only be performed by using cytological samples. Many groups have successfully performed EGFR testing on cytology samples, using Sanger sequencing, Pyrosequencing Mutation Analysis and PCR-based Cobas EGFR and KRAS Mutation Test kits. However, only a few of these studies have compared cytological specimen with histological specimen or blood specimen from the same patient [6]. Currently, we report EGFR mutation testing in cytological specimens of 291 non-small cell lung carcinoma cases using the amplification refractory mutation system (ARMS) in our institute. In this study, the matched tissue or blood samples were tested for EGFR mutations, in some cases, as comparison. As far as we know, the sample size of EGFR mutation analysis using cytologi-

cal material and the ARMS technique from a single institute is the largest in the literature.

Materials and methods

Case selection

EGFR mutation status was studied in 291 advanced NSCLC patients diagnosed at the Pathology Department, West China Hospital of Sichuan University from July 2013 to June 2015. The cytological diagnoses were rendered based on the cytomorphologic features and immunocytochemical studies. Twenty-one cases had histological results to confirm the cytological diagnoses. All of the cytological specimens had available cell blocks for EGFR mutation tests.

Specimen preparation

For the fluid specimen, 8 mL fluid was centrifuged at 2,500 revolutions per minute for ten minutes. Supernatant fluid was poured out and cell sediment was retained. The direct smears were prepared with Papanicolaou stain and the cell sediment was fixed in 10% neutral-buffered formalin for one hour, and then wrapped in filter paper to make cell block. For the FNA specimen, the remainder of fine-needle aspirate was fixed in 10% neutral-buffered formalin for 1 hour after preparing direct smears, and then the fixed material was wrapped in filter paper to process a cell block. After standard tissue processing, the paraffin embedded cell blocks were sectioned at a thickness of 4 μm to 6 μm. The HE stained slides were reviewed for assessment of the tumor percentage before EGFR mutation analysis. At least twenty tumor cells should be found on one slide and the tumor cell percentage should be above 5%.

DNA extraction and EGFR mutation analysis

Twenty to thirty sections were cut for each cell block and microdissection was used to enrich tumor cells in the case of tumor cell percentage below 20%. Genomic DNA was extracted from the FFPE (formalin fixed paraffin embedded) samples. The QIAamp FFPE Tissue kit (Qiagen, Germany) was used to isolate genomic DNA according to the manufacturer's instructions. After deparaffinization with xylene, the samples were digested with proteinase K (Qiagen, Inc.) and Qiagen ALT buffer overnight at 56°C. After digestion, the DNA was extracted using QIAamp (Qiagen, Inc.) spin columns following the manufacturer's instructions.

In this study, the matched histological samples were obtained from five patients and the matched blood samples were taken from four patients. EGFR mutation analysis also was performed using the matched histological samples and blood samples. The method of DNA extraction from FFPE histological samples was the same as that of the FFPE cell blocks.

The blood samples were collected into venous blood collection tubes using EDTA (ethylenediaminetetraacetic acid) as anticoagulant. The samples were mixed thoroughly and plasma was isolated by centrifugation at approximately 2,000 revolutions for 10 minutes (at 4°C or room temperature for pre-chilled samples) within two to four hours after sample collection. The samples were not thawed until the time of processing. Then ctDNA was extracted using the ctDNA Extraction Kit (Amoy Diagnostics, China).

Amplification Refractory Mutation System (ARMS) was used for EGFR mutation analysis. The EGFR Mutations Detection Kit (Amoy Diagnostics, China), which has been approved by State Food and Drug Administration (SFDA) in mainland China for clinical use, allows the detection of twenty-nine known recurrent mutations in EGFR exons 18-21, such as L858R in exon 21, L861Q in exon 21, T790M in exon 20, S768I in exon20 and 3 insertions in exon 20 (but not distinguishing them), G719X in exon 18 (G719S, G719A or G719C, but not distinguishing them) and deletions in exon 19. The EGFR mutation analysis was carried out for all of the samples according to the manufacturer's instructions. A BIO-RAD CFX96 machine was used for the PCR reactions. The results were considered as positive if one or more of the twenty-nine activating EGFR mutations were detected.

Statistical analysis

Chi-square test or continuity correction Chi-square test were applied to explore the association between clinicopathological variables and EGFR gene mutation status using SPSS software (SPSS Statistics 18.0; SPSS Inc.,

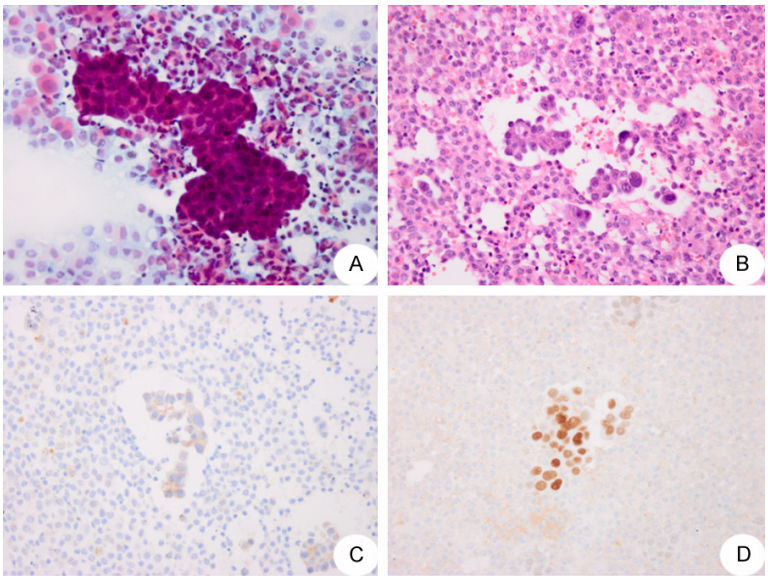

Figure 1. Representative sample of pleural fluid specimen of pulmonary adenocarcinoma: A. Conventional smear (Papanicolaou stain ×400); B. Cell block section (HE stain ×400); C. The tumor cells were positive for NapsinA (EnVision ×400); D. The tumor cells were positive for TTF-1 (EnVision ×400).

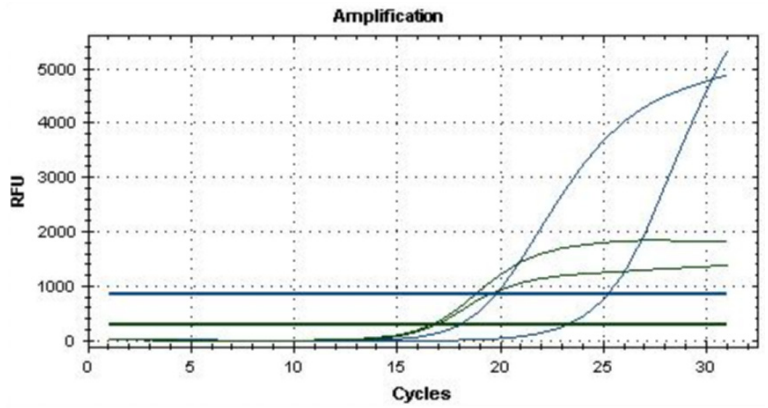

Figure 2. L858R point mutation and T790M point mutation were both detected in the pleural fluid specimen from a patient with pulmonary adenocarcinoma (ARMS).

Chicago, Ill). Two-sided *p*-value <0.05 was considered statistically significant.

Results

Patient information

291 NSCLC patients were included in this study (144 men and 147 women). The median age of the patients at the time of diagnosis was 62 years, which ranged from 21 to 88 years. Informed consent was obtained from all participants or from their guardians. These 291 cases included 281 of adenocarcinomas (**Figure 1**) and 10 of squamous cell carcinomas. There were 244 of pleural fluids, 7 of pericardial fluids, and 2 of peritoneal fluids. There were 38 of FNA cases which consisted of 30 cervical lymph nodes aspirations, 6 of endobronchial ultrasound-guided transbronchial mediastinal lymph nodes aspirations (EBUS-TBNA), one of a lung mass and ilium mass aspiration.

EGFR mutations in NSCLC samples

289 samples were successfully tested while the results of two samples were invalid because of the bad DNA quality. EGFR mutations were detected in 131 cases (130 adenocarcinomas and one squamous cell carcinoma). The mutation rate was 45.3% (131/289). The detected EGFR mutations included L858R point mutation in exon 21 (54 cases), deletions in exon 19 (69 cases), G719X point mutation in exon 18 (1 case), S768I point mutation in exon 20 (1 case), L861Q point mutation in exon 21 (1 case), and an insert mutation in exon 20 (1 case). In four cases, more than one type of mutation was detected. The compound mutations included L858R combined 19del (1 case), L858R combined T790M (1 case, **Figure 2**), and G719X combined S768I (2 cases). The EGFR mutation status related to the different samples is listed in **Table 1**. For the FNA cases, EGFR mutations were identified in 17 cases including 12 cases of cervical lymph nodes, 4 cases of mediastinal lymph nodes, and 1 of ilium mass. The mutation rate was 44.7%. For the effusions, one pericardial fluid was positive for 19del and one peritoneal fluid was positive for L858R. The other mutations were detected

Table 1. The EGFR mutation status related to the different samples

Mutation status	FNA	Effusion	Total
Wild type	21	137	158
L858R	3	51	54
19del	12	57	69
G719X	1	0	1
S768I	0	1	1
20Ins	0	1	1
L861Q	0	1	1
L858R+19del	0	1	1
L858R+T790M	0	1	1
G719X+S768I	1	1	2
Invalid results	0	2	2
Total	38	253	291

Abbreviation: FNA, Fine needle aspiration.

in pleural fluids and the mutation rate was 45.8%. The incidence of EGFR mutations according to different age groups is shown in **Figure 3**. The EGFR mutation rate was highest in ages 50-59 years (57.9%), followed by that of patients under 30 years old (57.1%). It was lowest in ages 30-39 years (38.5%), followed by that of patients beyond 70 years old (39.2%). However, there was no statistical relationship between the age groups and EGFR mutations. The EGFR mutation status as related to the different clinicopathological characters is listed in **Table 2**. EGFR mutation status only had a significant difference between males and females ($P<0.005$). The age at diagnosis, the sample type, and histological type were not associated with EGFR mutations.

In this study, matched histological samples were obtained from five patients, of which the pathological diagnoses were consistent with the cytological diagnosis. Among them, one was bronchosopic biopsy, two were core needle biopsies from lung, and the rest were resection specimens of lung or lymph node, respectively. EGFR mutation analysis, using histological samples and cell block samples, yielded concordant results for three patients. For the remaining two patients, EGFR detections on the histological samples demonstrated L861Q and 19del, respectively, whereas the tests using cell blocks showed wild-type genes. Among four patients who provided blood samples, EGFR detection results were the same as that of cell blocks in three patients. In one patient, the detection using cell block revealed 19del while the blood sample showed wild-type gene. The time intervals of EGFR detection between cell blocks and the matched histological or blood samples ranged from 3 days to 3 years. The detailed information is listed in **Table 3**.

Discussion

Detection of EGFR gene mutation is critical for individualized treatment of lung cancer [7]. EGFR mutation can be considered as a predictor for tyrosine kinase inhibitor (TKI) treatment which is efficient for NSCLC [8, 9]. EGFR gene mutation detection using DNA extracted from surgical specimen is currently the most sensitive and reliable method [10]. However, some NSCLC patients which have advanced stage IIIB or IV at the time of diagnosis, lost their opportunity for surgical treatment. In addition, a portion of elderly patients may not be able to tolerate surgery or invasive examinations, such as pneumocentesis biopsy or bronchoscopic biopsy. Under the above mentioned circumstances, the unavailability of the tissue samples from NSCLC patients limits the clinical application of EGFR gene mutation testing.

Malignant serous effusion and distant metastasis are common manifestations of advanced NSCLC. Puncture of the serous cavities is relatively easy, with less pain, and has a simultaneous treatment effect for the patients. For peripheral metastatic lesions that are unavailable for resection, tumor cells can be collected through fine-needle aspiration biopsy. Fresh effusion samples and FNA samples can yield high-quality DNA for detection of oncogenic mutations [2, 11], but it is difficult to determine whether there are sufficient tumor cells in the samples which may increase the risk of detection failure. In this study, the above mentioned two types of cytological samples were used as study subjects and cell block was prepared for each case. The existence and quantity/content of tumor cells were evaluated using the sections of cell blocks to ensure the reliability of detection results.

Using the ARMS technique, EGFR gene status was successfully detected in 289 out of 291 cytological samples in this study. The ARMS PCR is recommended by multiple medical associations [12]. Compared to the direct sequenc-

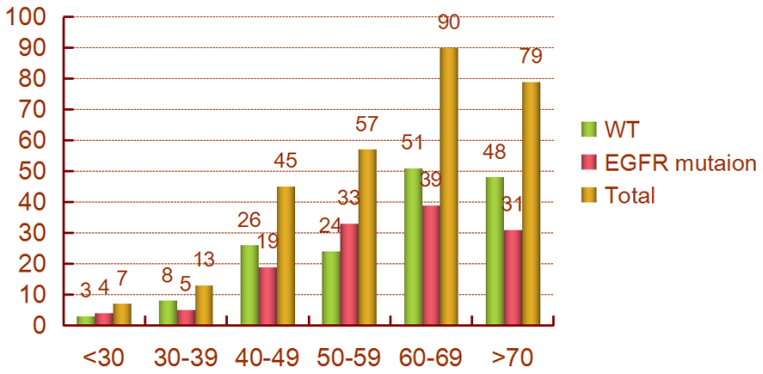

Figure 3. The incidence of EGFR mutations in non-small cell lung carcinoma patients according to different age groups (at diagnosis). WT: Wild type.

Table 2. The clinicopathological characteristics of non-small cell lung carcinoma patients with EGFR mutations

Characters	N	EGFR mutations	χ^2	*P*
Age, years			0.093	0.760
<62	137	60 (43.8%)		
≥62	152	71 (46.7%)		
Gender			8.723	0.003
Male	144	45 (31.3%)		
Female	145	86 (59.3%)		
Samples			0.002	0.961
FNA	38	17 (44.7%)		
Effusion	251	114 (45.4%)		
Histological type			1.622	0.203
Adenocarcinoma	279	130 (46.6%)		
Squamous ca	10	1 (10%)		

Abbreviations: N, Patients number; FNA, Fine needle aspiration; Squamous ca, Squamous cell carcinoma. 62 was the Median age at diagnosis. Two samples with bad DNA quality were not included.

Table 3. The comparison of EGFR mutation status in cell blocks and the matched histological or blood samples

	EGFR detection results			
Cases	Cell blocks	Histological samples	Blood samples	Time intervals
1	W	L861Q	NA	6 m later
2	19del	19del	NA	10 m later
3	W	W	NA	9 m before
4	W	W	NA	6 d later
5	W	19del	NA	6 m later
6	19del	NA	W	3 y later
7	19del	NA	19del	5 m later
8	19del	NA	19del	11 m later
9	W	NA	W	3 d before

Abbreviations: W, Wild type; NA, Not available; m, month; d, day; y, year. The time intervals were based on the tests of cell blocks.

ing method, this technique has better sensitivity and specificity, and has a higher success rate for wax-embedded samples [13, 14]. Sun and Liu revealed that tumor cell content higher than 25% in the tissue sample was the prerequisite for successful detection [6, 13]. However, using the microdissection method, the tumor cell content required for detection was reduced to a lower level (only >5%) in this study. The positive rate of EGFR mutation in 289 cytological samples was 45.3%, similar to the positive rate obtained from the large cohort of routine histological samples (49.4%) [15]. In addition, the vast majority mutation types were 19del and L858R in both cytological and histological samples. These facts demonstrated that cell block samples could serve as an effective substitute for histological samples for EGFR detection, similar to the previous reports [16].

In this study, among five patients whose histological samples were available as control, EGFR mutations (L861Q and 19del) were found in histological samples of two patients, respectively, but not in the matched cell block samples, suggesting heterogeneity of EGFR mutations [17]. Other studies have found that EGFR mutation heterogeneity manifested in two aspects: one was the inconsistency between the primary tumor and metastatic lesions, which might explain the inconsistent responses of primary and metastatic lesions to TKI treatment in certain patients with metastatic lesions [18, 19]; another was the intratu-

moral heterogeneity of EGFR mutations [20-22].

Among four patients whose blood samples were available for EGFR mutation detection, one patient was found to have the 19del in the cell block sample but not in the blood sample. In addition to the heterogeneity, another possible reason could be the insufficient number of tumor cells in the blood. This result also indicated that pre-detection morphological evaluation of the quantity/quality of tumor cells was very important. Besides the aforementioned reasons, tumor therapy such as chemotherapy might change the EGFR gene mutation status [23]. However, the matched histological and blood samples were still limited and the treatment information for these patients was unclear in the current study, so more corresponding samples and clinical information were planned to be collected for the next EGFR mutation analysis.

Using cell blocks for detection has a variety of advantages, including high success rate, reliable results, and relatively high clinical practicability. Cell block samples can be an effective substitute for histological tissue samples. In addition, the paraffin embedded cell blocks can be stored long-term for future immunohistochemical (IHC) staining or fluorescence in situ hybridization (FISH) [24]. It is highly necessary to assess the morphology and quantity of tumor cells before detection in order to improve the positive rate. The microdissection method is suggested to enrich tumor cells in the case of tumor cell percentage <20%. Considering the existence of tumor heterogeneity, we still recommend that both histological and cytological samples should be collected for examination, if possible, to ensure individualized precise treatment.

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Disclosure of conflict of interest

None.

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