Original Article
High concordance of EGFR mutation status between sputum and corresponding tissue specimens of late-stage lung cancers using amplification refractory mutation system-PCR

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Abstract: Background: To gain sufficient tumor tissue for EGFR mutations analysis is of prime clinical importance. Therefore, the objective of present study was to assess whether sputum is suitable for EGFR testing and to assess the consistency between sputum and tissue specimens. Methods: This analysis included 37 paired sputum and tissue specimens obtained from late-stage lung cancer patients followed by EGFR analysis using the ARSM-PCR method, and 11 sputum specimens of COPD were added as negative control. Results: 35 out of 37 (94.6%) patients with lung cancer were found to have tumor cells in the sputum specimen using the LCT method and extracted adequate DNA for EGFR testing. In contrast, only five out of 11 cases of COPD (45.5%) were isolated with sufficient DNA (P<0.001). Among sputum testing, higher EGFR mutation was significantly associated with NCSLC and ADC (40%, 10/25), stage IV (45.5%), and non-smoking status (43.8%). Compared with corresponding tissue specimens, the accuracy, specificity, and sensitivity of EGFR mutation analysis in sputum specimens were demonstrated as 97.1%, 96%, and 90.9%, respectively. Interestingly, a significantly high frequency of L858R in exon 21 was found (P<0.001) in all types of EGFR mutants both on sputum and tissue specimens. Conclusions: The findings of the current study suggest a high concordance between sputum and tissue specimens, reflecting the important potential of sputum specimens for EGFR mutant detection in patients with late-stage lung cancer using the ARSM-PCR method.

Keywords: Sputum, epidermal growth factor receptor (EGFR), amplification refractory mutation system-PCR (ARMS-PCR), liquid biopsy, lung cancer

Introduction
Lung cancer has the highest mortality rate of all cancers worldwide and remains a major health threat for the foreseeable future both in men and women [1]. Recently, based on remarkable advances in the understanding of oncogenic drivers which served as therapeutic targets, it is fully accepted that adenocarcinomas (ADC) and non-small cell lung cancers (NSCLC) should be routinely tested for the major genomic alteration-epidermal growth factor receptor (EGFR) mutations-to identify those who are eligible for treatment with EGFR tyrosine kinase inhibitors (TKIs) suggested as a first line drug [2]. Furthermore, nearly two thirds of patients with lung cancers suffered advanced-stage disease at the time of diagnosis, and most of them lost the chance to undergo surgical operation. Therefore, adequate tumor samples taken in a proper form are clinically important for a complete pathologic diagnosis including tumor histological subtyping and analysis of genetic alterations. Consequently, pathologists are required to try their best to test for EGFR mutations.

Generally, diagnosis and EGFR testing have been performed by using tumor tissues obtained by small biopsy or surgical resection, which is recommended as the ‘gold standard’. In addition, some diagnoses of lung cancer are based on cytological specimens, such as fine-needle aspiration (FNA), pleural fluid, bronchial
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washing, as well as brushing specimens. It can be challenging to gain sufficient tumor tissue for EGFR testing, since biopsy and cytological samples are small or are prioritized for histological diagnosis. On the other hand, all of the techniques mentioned above might not be accompanied by patients with severe complications because of invasive diagnostic procedure.

For decades, physicians started with sputum to locate intra-thoracic lesions, including lung cancer. Sputum collection is non-invasive, and easy to obtain routinely each day [3]. In some critical cases, sputum might represent the only material available for pathological diagnosis. However, the concordance of molecular analyses between the formalin-fixed, paraffin embedded cell blocks derived from FNAs and pleural fluid with histological specimens is well established [4, 5]. But, few of previous studies have analyzed sputum specimens by the amplification refractory mutation system (ARMS)-PCR [6, 7].

To explore the new material available for EGFR testing, sputum specimens were investigated to assess whether they are suitable for the molecular analysis of EGFR mutation status. Furthermore, the objective of the current study was to assess the correlation of EGFR status between sputum with tissue specimens from the same patient with late-stage lung cancers.

Materials and methods

Clinical samples

The department of Pathology at West China Hospital of Sichuan University is the biggest centralized laboratory for EGFR testing in South-West China, and conformed to both Pathology Quality Control Center of China and College of American Pathologists (CAP). Between September 2016 and September 2017, sputum samples from patients with late-stage lung cancer were diagnosed and assessed the cellularity by two experienced cytopathologists using liquid-based cytological technology (LCT) preparation, followed by storage at 4°C when cancer cells were observed. From this cohort, 37 sputum specimens, including 35 cases of lung cancer, two of atypical cells, and their corresponding histological specimens without any prior targeted therapy were collected for analysis of EGFR status. In contrast, 11 sputum specimens with COPD confirmed by histology were selected to EGFR testing as well. The cytological and histological diagnoses of these cases were made according to the 2015 WHO classification. Our study was approved by the ethics committee of West China Hospital and carried out following the local ethical guidelines.

Pre-analytic preparation

Sputum specimens were collected into a 1.5 ml Eppendorf tube, followed by centrifugation at 3,000 rpm for 3-5 minutes. After remove of the soluble supernatant, the tube was incubated in a water bath at 60°C for 3-5 minutes. Genomic DNA was then isolated as soon as possible.

DNA isolation

DNA was extracted from sputum specimens and their corresponding tissue samples by using QIAamp DNA FFPE Tissue Kit (Qiagen, German), according to the manufacturer’s instructions. ScanDrop 200 spectrophotometer (Analytik Jena, Germany) was used to determine the concentration and quality of DNA by calculating optical absorbance at wavelength 260 nm and the ratio of wavelengths at 260 nm to 280 nm. The ratio of 1.8±0.2 was considered as qualified DNA. Each extract was manipulated to a final volume of 50 ml with the concentration of 1 ng/μl for further EGFR mutation analysis.

EGFR mutation analysis

EGFR status of all specimens mentioned before was carried out using Amplification Refractory Mutation System-PCR (ARMS-PCR) technology on a BIO-RAD CFX96 machine (BIO-RAD, American), which is fully evaluated on histological tissue. The ADx EGFR Mutations Detection Kit (Amoy Diagnostics, China) was subjected to this procedure, which was approved for clinical application by the State Food and Drug Administration in China. Therefore, 29 of known recurrent mutations in EGFR exons 18-21, which include G719X (but not discriminating among G719S, G719A, and G719C) in exon 18, 19 deletions (including E746_A750del, L747_P753>S, E746_T751>I, E746_T751del, E746_T751>A, E746_S752>A, E746_S752>V, E746_S752>D, L747_A750>P,
EGFR testing on paired sputum and tissue

Table 1. Characteristic of 37 patients with paired sputum and tissue specimens

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of patients n=37 (%)</th>
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<tbody>
<tr>
<td>Age, years</td>
<td>Median (range) 67 (45-89)</td>
</tr>
<tr>
<td>Sex</td>
<td>Male 29 (78.4%) Female 8 (21.6%)</td>
</tr>
<tr>
<td>Smoking status</td>
<td>Smoker 19 (51.4%) Non-smoker 18 (48.6%)</td>
</tr>
<tr>
<td>Stage</td>
<td>III 26 (70.3%) IV 11 (29.7%)</td>
</tr>
<tr>
<td>Diagnoses on sputum specimens</td>
<td>ADC 20 (54.1%) SCC 8 (21.6%) NSCLC 5 (13.5%) SCLC 2 (5.4%) Atypical cells 2 (5.4%)</td>
</tr>
<tr>
<td>Resource of histologic specimens</td>
<td>CT-guided percutaneous transthoracic biopsy 19 (51.4%) Flexible bronchofiberscopic biopsy 12 (32.4%) Surgical resection 6 (16.2%)</td>
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Abbreviations: ADC, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.


Statistical analysis

The cytology results were compared with corresponding tissue samples individually and classified as true positive, true negative, false positive, and false negative tests. Sensitivity [(true positive/total positive) × 100%], specificity [(true negative/total negative) × 100%], and accuracy [(true/(true + false) × 100%)] were determined. Statistical Package for Social Science version 21.0 for windows (SPSS Inc., Chicago, IL, USA) software was used to analyze the different groups. Differences between sputum and tissue specimens were analyzed using Chi-square test. If there were five or fewer observations in a group, the Fisher exact test was performed. P value less than 0.05 was considered as statistically significant.

Result

Patient characteristic

A total of 48 patients (36 men and 12 women) were involved in the present study. Among them, 37 patients (29 men and eight women) with late-stage lung cancers were collected both sputum and tissue specimens for further analyses. The clinicopathological features of 37 patients are summarized in Table 1. Another 11 patients (seven men and four women) suffered with chronic obstructive pulmonary disease (COPD) confirmed by physicians and histological examinations.

As shown in Table 1, the median age of the cancer patients at the time of diagnosis was 67 years (45-89 years). Of the 37 sputum specimens, 20 were diagnosed as adenocarcinoma (ADC) (Figure 1), 8 were squamous cell carcinoma (SCC) and two were small cell carcinoma. However, five malignant cases didn’t show differentiated morphologic features on cytology and termed to “non-small cell lung cancer, not otherwise specified (NSCLC, NOS)”. The remaining two cases observed several condensed cells on the LCT preparation and were defined as “atypical cells”. Therefore, 35 out of 37 (94.6%) patients with lung cancer were found with tumor cells in sputum specimens using LCT method. Whereas, analysis of the remaining two cases failed to detect certain tumor cells.

Four NSCLCs and two “atypical cells” based on cytology were demonstrated as ADC on tissue specimens by using the immunohistochemistry (IHC) diagnostic panel (thyroid transcription factor 1 [TTF-1] positive, CK7 positive, and p63 negative). However, another one NSCLC still considered as “not otherwise specified” fol-
EGFR testing on paired sputum and tissue

Figure 1. Representative cases of (A) 1% and (B) more than 90% of tumor cells observed using liquid-based cytological technology (LCT). Microphotographs of (C) and (D) were corresponding tissue specimens of (A) and (B), respectively.

Figure 2. Incidence of EGFR exons 18-21 mutations in late-stage lung cancers according to histological types in (A) sputum specimens and (B) tissue specimens. The mutation frequency was highest in NSCLC, followed by ADC in both.

Lowed by flexible bronchofiberscopic biopsy and IHC examination. Consistent with sputum samples, eight histological specimens were determined as SCC. Two SCLC were confirmed by two experienced pathologists and IHC procedure (PanCK, CgA and/or CD56 positive, and p63 negative).

Cellularity of sputum specimens and efficiency of DNA isolation

A total of 48 sputum samples (37 of lung cancers and 11 of COPD) was examined by two experienced cytopathologists on liquid-based cytological technology (LCT) for the presence of cells available for DNA isolation. Among 37 lung cancers, cytological examination showed that the percentage of malignant cells on sputum specimens ranged from 1% to 90% in 35 of cases (94.6%, 35/37). As mentioned before, the remaining two lung cancers did not contain malignant cells, followed by failure of DNA extraction. No matter how cellular the sample was, all malignant cases were extracted adequate DNA successfully for EGFR mutation analysis and this ranged from 7.4 to 686.9 ng/μl (average 72.8 ng/μl).

For the 11 sputum samples from COPD, a few of inflammatory cells (lymphocytes and/or neutrophils) and bronchial epithelium were found. Furthermore, significantly fewer cases of COPD (45.5%, 5/11) were isolated sufficient DNA when compared with malignant cases.
EGFR testing on paired sputum and tissue

From this cohort, there were 3 types of EGFR mutations observed (Figure 3A). In line with the literature, L858R in exon 21 (70%, 7/10) and deletions in 19 exon (20%, 2/10) accounted for the vast majority of the mutations. However, the remaining mutation referred to the insertions in exon 20. Interestingly, a significantly high frequency of L858R in exon 21 was found (P<0.001).

**EGFR mutation analysis on corresponding tissue specimens**

On the other hand, 37 corresponding tissue specimens were determined to have the EGFR mutation upon analysis using ARMS-PCR. As shown in Figure 2B, since four NSCLCs and two “atypical cells” diagnosed on LCT were confirmed as ADC on histological examinations, the higher EGFR mutation rate in ADC (38.5%, 10/26) was observed, when compared with sputum specimens. However, one case corresponding with “atypical cells” exhibited deletions in exon 19. There was no association between EGFR mutation and the nature (CT-guided percutaneous transthoracic biopsy vs. flexible bronchofiberscopic biopsy; biopsy vs. surgical resection) of the specimens used for the mutation analysis. EGFR mutation frequency reached 40.7% (11/27) in NSCLC and ADC.

Consistent with sputum samples, three kinds of EGFR mutations were determined (Figure 3B), including L858R in exon 21 (66.7%, 8/12), deletions in 19 exon (25%, 3/12) and insertions in exon 20 (8.3%, 1/12). Compound EGFR mutations were not seen in both sputum and tissue samples.

### Table 2. Correlation of EGFR status between sputum and tissue specimens from 35 paired samples

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Concordance of EGFR testing between sputum and corresponding tissue specimens

A total of 35 paired sputum and tissue specimens were conducted to EGFR exons 18-21 mutations analyses using the same methodology. As shown in Table 2, positive results obtained from sputum samples were all in agreement with corresponding tissue samples. Of the one non-concordant case, the mutation of L858R in exon 21 was obtained in tissue specimen, but not in the corresponding sputum specimen.

In summary, taking results from tissue specimens as the gold standard, no false positives were observed in sputum specimens. Therefore, the accuracy, specificity and sensitivity of EGFR mutation analysis in sputum specimens by using AMRS-PCR method were demonstrated as 97.1%, 96%, and 90.9%, respectively. In addition, there was no statistical difference between sputum and tissue specimens on EGFR mutation frequency in all histological subtypes (P>0.05).

Discussion

Since more than half patients suffered from late-stage lung cancer at the time of diagnosis, it still remains critically important to obtain enough samples for molecular analysis, including EGFR testing. In the last five years, several minimally invasive techniques have been introduced to improve the situation, such as defining cell free DNA (cfDNA), exosomes, circulating tumor cells (CTCs) and DNA (ctDNA), etc. However, most of them were determined in blood, serum, or plasma, which is referred as liquid biopsy [4, 8-10]. Unfortunately, previous studies showed that the sensitivity of these methods only reached about 40%-60% on the patients with late-stage adenocarcinoma, even though they have higher specificity and accuracy [11]. Actually, liquid biopsy analysis could be performed on almost all body fluids, including blood products, pleural effusion, ascites, urine, as well as sputum. However, few previous reports explored the possibility of sputum specimens for EGFR mutation analysis, and none of them was conducted by ARMS-PCR technology [6]. Thus, it remains unclear whether the EGFR status in sputum specimens can reflect the profiles revealed by tissue specimens. Therefore, the current study focused on the usefulness of the sputum specimen for EGFR mutation analysis using ARMS-PCR and compared with corresponding tissue specimens. The results provide some exciting and promising data.

From this cohort, EGFR mutation analysis on sputum specimens was in agreement with previous findings on histological specimens that (1) the mutant frequency (>40%) was consistent with an audit findings of tissue specimens in China [12], (2) they were highly associated with ADC and NSCLC, (3) higher mutant EGFR was statistically associated with non-smoker, and (4) more than 90% of mutant type was either L858R in exon 21 or deletions in 19 exon. Altogether, sputum specimens are useful material for EGFR testing.

More importantly, the accuracy of sputum EGFR testing reached about 97% in informative patients with late-stage lung cancer. Additionally, specificity and sensitivity were achieved about 96% and 91%, respectively. In detail, all positive results obtained from sputum EGFR mutation analysis were confirmed as true positive cases. Only one negative result was demonstrated as mutant-type EGFR. In another word, positive detections of sputum EGFR testing are reliable, whereas negative results need more careful explanation.

Interestingly, we detected obviously higher proportions of L858R in both sputum (70%) and tissue (66.7%) samples. To the best of our knowledge, the frequency of L858R in exon 21 and deletions is nearly equal to each other on histological findings [12]. We speculate that it may due to two possibilities: (1) limitation of the number of cases herein; and (2) location (proximal bronchus) susceptibility. It’s reasonable for us to suggest that L858R might be associated with an aggressive behave with involvement of the central airway compartment. This hypothesis needs enlargement of the case number and further experimental support in future study.

In our routine practice, malignant cells might not be found in some sputum obtained from lung cancer patients. Maybe that’s why previous exploration couldn’t gain satisfied results. In the present study, EGFR mutation analysis was focused on malignant sputum samples following LCT examination, resulting in exciting accuracy. No tumor cells detection in two cases
of “atypical cells” and partial of COPD were failed to DNA isolations. Then, containing tumor cells is considered as inclusion criteria in clinical practice, since significantly higher success rate of DNA extraction and concentration in positive cases was obtained.

Another reason of lower sensitivity in sputum EGFR mutation analysis previously might be due to the technology used in test. The ARMS-PCR used here was approved by the State Food and Drug Administration of China (CFDA) and European Conformity (CE). However, to our knowledge, it has not yet been approved by the US Food and Drug Administration. As indicated by the manufacturer and also confirmed in our routine clinical application, the tech was capable of detecting EGFR mutation at a frequency of approximate 1% of tumor cells [12-15]. Consistent with tissue findings, the proportion of tumor cells in each sputum specimen was estimated and this ranged from 1% to 90%. Particularly, even 1% of tumor cells seen in LCT, DNA isolation and EGFR testing were succeed in present study (Figure 1A). That means once malignant cells are found in LCT, whatever the proportion, the remaining sputum specimen could proceed to EGFR testing.

In conclusion, our data from these paired sputum and tissue specimens reflect high concordance, and also demonstrate the satisfied accuracy, specificity, and sensitivity of sputum EGFR testing. Finally, our findings support that sputum obtained by noninvasive approach could be conducted to detect EGFR-mutations in patients with late-stage lung cancer. However, we do not suggest using sputum specimens as routine procedure for molecular analysis, but rather storing them temporarily as an alternative in case tissue samples become unavailable.

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Disclosure of conflict of interest

None.

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References

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