Original Article

Evaluation of liquid based cytology in detection of EGFR mutation in NSCLC by large samples

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Background: Cytology samples are the main resources to detect driver oncogene alterations for advanced lung cancer patients. To explore the value of liquid-based cytology in the detection of epidermal growth factor receptor (EGFR) mutation in non-small cell lung cancer (NSCLC), we analyzed data from a large cohort of EGFR mutation-positive patients.

Methods: We analyzed the clinicopathological characteristics of 8,029 NSCLC cases tested for EGFR mutation by liquid-based cytology specimens and 1,934 NSCLC cases tested by formalin-fixed and paraffine-embedded (FFPE) samples in the Shanghai Pulmonary Hospital from September 2015 to December 2019. Before detection, we evaluated the number of tumor cells in the liquid-based cytology slide, and samples with more than 50 tumor cells and visible sediment were selected for DNA extraction after centrifugation.

Results: The positive rate of EGFR mutation in liquid-based cytology-tested cases was 47.18%, higher than the 41.37% tested through FFPE sample (P<0.01). Accordingly, the mutation rate of EGFR in adenocarcinoma (AC) and NSCLC was higher than that of the FFPE sample (60.01% *vs.* 54.15%, P<0.01; 30.54% *vs.* 21.99%, P<0.01). The positive rate of EGFR mutation in pleural effusion was 62.67%, which was the highest rate among liquid-based cytology sample t (P<0.01).

Conclusions: Using quality control and standard procedure, it was found that liquid-based cytology specimen testing is a convenient and reliable method of EGFR detection, as validated by analysis of a large cohort. EGFR mutation detection should also be carried out in NSCLC patients diagnosed by cytology more than in AC patients.

Keywords: Non-small cell lung cancer (NSCLC); cytology specimens; liquid-based cytology; epidermal growth factor receptor (EGFR); drive mutation

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Introduction

The detection of the epidermal growth factor receptor (EGFR) mutation is crucial for the treatment of non-small cell lung cancer (NSCLC) patients. Numerous clinical trials have shown that the progression-free survival (PFS) of NSCLC patients with EGFR mutation and treated with tyrosine kinase inhibitors (TKI) is significantly prolonged (1,2). Approximately two-thirds of NSCLC patients in advanced stages lose the chance of operation, and these patients can only be diagnosed by biopsy or cytology samples (3). In addition to pathological diagnosis, detection of driver oncogene mutation including EGFR target

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should be conducted. An increasing number of laboratories are applying cytology specimens, including liquid-based cytology specimens, cell blocks, and smears, even plasma to molecular detection, although formalin-fixed and paraffine-embedded (FFPE) specimens remain the primary sample type used (4,5). Liquid-based cytology is convenient, easy to operate, and reliable in molecular testing; however, the positive rate of liquid-based cytology is inconsistent in many reports (6-8). Few reports on the analysis of large samples have been published, and there is also no standard for the procedure of EGFR detection by liquid-based cytology samples, especially for the evaluation of tumor cells before detection. In our previous research, we explored using a standard quality control process of liquid-based cytology specimen testing for EGFR detection (9). Here, to verify the feasibility of using liquid-based cytology to detect the EGFR mutation gene, the EGFR status of 8,029 samples was tested using liquid-based cytology under this standard quality control process. Another 1,934 cases of EGFR mutation were analyzed by using FFPE samples. From this, we attempted to establish a standardized procedure of liquid-based cytology for EGFR molecular detection. We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/jtd-20-2750).

Methods

Case selection

In this study, the EGFR mutation status of 9,963 cases with informed consent were tested, including 8,029 liquid-based cytology samples and 1,934 FFPE samples from September 2015 to December 2019 in Shanghai Pulmonary Hospital. Age, gender, and pathological classification of lung cancer was compared in the 9,963 cases. EGFR mutation features were compared between liquid-based cytology samples and FFPE samples. Pathological diagnosis according to cytology was divided into three subtypes: adenocarcinoma (AC), squamous cell carcinoma (SCC), and NSCLC. Cytology samples included percutaneous fine needle aspiration (FNA), pleural effusion (PE), sputum, bronchial brushing, bronchoalveolar lavage fluid (BALF), and endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) samples. FFPE samples included biopsy specimens and surgically resected specimens. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Ethical approval for the study was obtained from the ethical committee of Shanghai Pulmonary Hospital (approval number: L20-331Y).

Quality control of EGFR mutation detection of liquid based cytology specimens (8)

(I) We evaluate the number of tumor cells in the liquid based cytology slide before EGFR mutation detection. Samples satisfied with more than 50 tumor cells and visible sediment were extracted DNA after centrifugation. If the sample fails to meet this standard, we will suggest the physicians or patients to resample to avoid invalid EGFR detection. (II) We estimated the diameter of precipitation of liquid based cytology specimens. DNA and RNA which can be used to detect anaplastic lymphoma kinase (ALK) and c-ros oncogene 1 receptor tyrosine kinase (ROS1) genes were extracted at the same time, when the diameter was between 2–5 mm. For specimens with precipitation diameter >5 mm, the residual cell pellet was resuspended in the solution and then was divided equally into 2–1.5 mL centrifuge tube. DNA and RNA were extracted separately. The workflow model is listed in *Figure 1*.

DNA extraction process of liquid based cytology

(I) Liquid based cytological specimens stored in ThinPrep (hologic Gen probe) preservation solution were centrifuged for 12,000 r/min, 3 minutes, and precipitation was transferred to 1.5 mL centrifuge tube. (II) The total DNA was extracted using an AmoyDx DNA Kit (Amoy Diagnostics Co, Xiamen, China) according to the manufacturer's instructions. The quality and quantity of the DNA was measured on an FLx800 Spectrophotometer (BioTek Instruments, Inc., USA).

DNA extraction process of FFPE samples

(I) For FFPE samples, wax block was cut into 3 rolls (10 μm thick/roll). And then put rolls into 1.5 mL centrifuge tube. (II) Add 1 mL dimethylbenzene into centrifuge tube to dewax (56 ℃, 15 min), and then centrifuge for 12,000 r/min, 3 minutes, repeat this step. (III) Discard the supernatant, neutralize the residual dimethylbenzene with anhydrous ethanol. (IV) The total DNA was extracted using an AmoyDx DNA Kit (Amoy Diagnostics Co, Xiamen, China) according to the manufacturer's instructions. The quality

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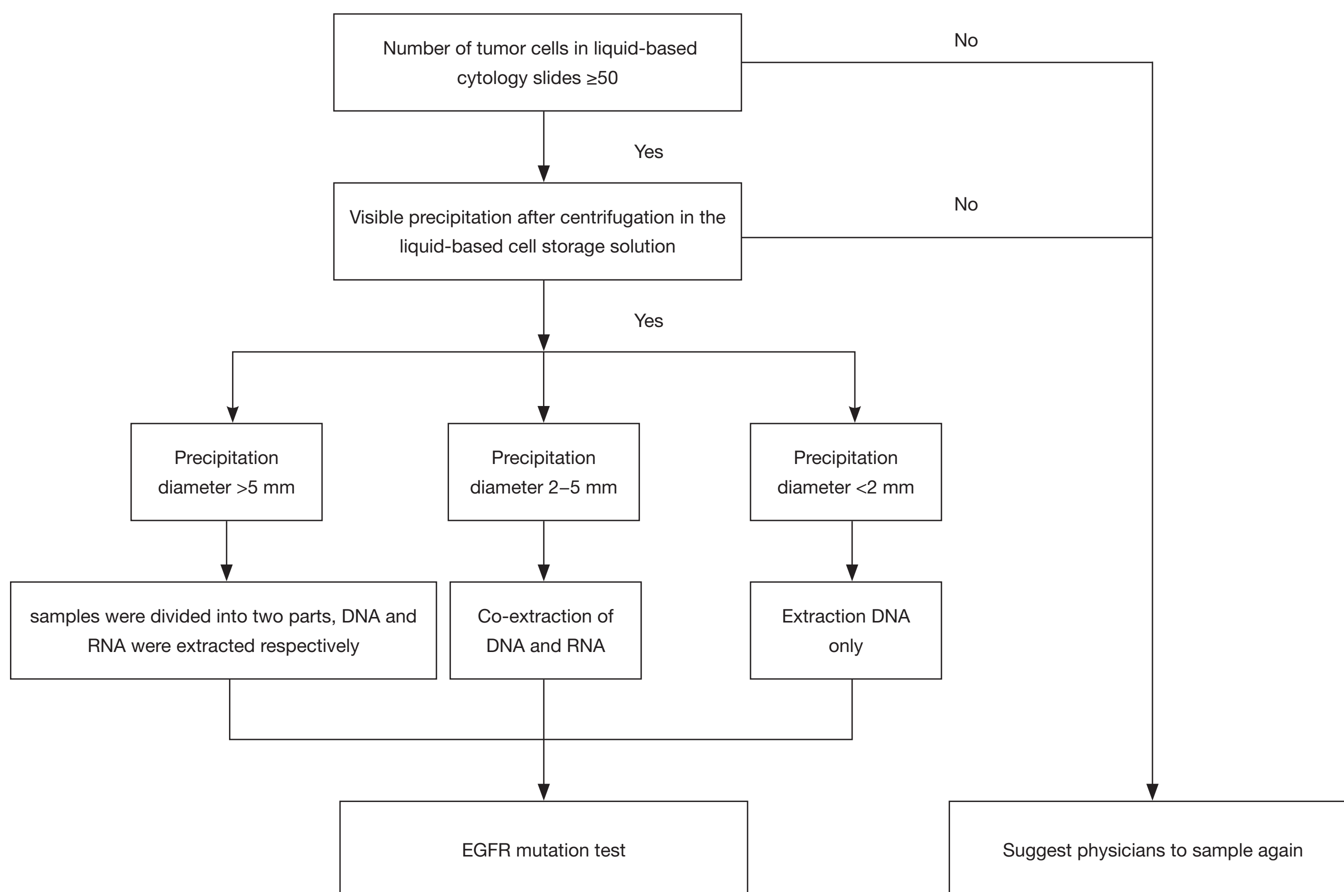

Figure 1 Processes of EGFR quality control tested by liquid-based cytology samples. EGFR, epidermal growth factor receptor.

and quantity of the DNA was measured on an FLx800 Spectrophotometer (BioTek Instruments, Inc., USA).

EGFR mutation test

The EGFR gene were detected by ARMS-PCR using an ADx EGFR Gene Detection Kit (Amoy Diagnostics Co, Xiamen, China). The PCR amplification (95 ℃ for 5 minutes, 15 cycles of 95 ℃ for 25 seconds, 64 ℃ for 20 seconds, and 72 ℃ for 20 seconds; and then, 31 cycles at 93 ℃ for 25 seconds and 60 ℃ for 35 seconds) was performed. The status of the genes was detected on a Stratagene Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA, USA).

Statistical analysis

The statistical analyses were performed using SPSS software version 25.0 (IBM, Armonk, NY, USA). Continuous variables were compared using Mann-Whitney U tests. Variables displayed as percentages were compared with a χ^2 test. Differences with P<0.05 were considered significant.

Results

Study population and clinical pathological characteristics

In this study, 9,963 cases were enrolled. The age ranged from 19 to 94 years old, and the median age was 65 years old. EGFR mutation were detected in 4,587 cases, the positive rate was 46.04%. EGFR mutation rate was higher in 3,924 female patients (65.07%) than 6,036 male patients (33.68%) (P<0.01). The EGFR mutation rate of AC, NSCLC and SCC was 58.57%, 30.04% and 8.33% accordingly (P<0.01). In our study, 8,029 cases were detected by liquid based cytology specimens, accounting for 80.59%. The EGFR mutation rate of liquid based cytology

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Table 1 Clinical and pathological features of 9,963 patients with EGFR mutation tests

Clinical and pathological features	Case number (percentage)	Number of EGFR mutation cases (percentage of mutations)	χ^2	P
Age (media, range)	65 [19–94] years old	4,588 (46.05%)		
Gender			944.269	0
Male	6,039 (60.61%)	2,034 (33.68%)		
Female	3,924 (39.39%)	2,554 (65.07%)		
Pathological subtype			972.050	0
Adenocarcinoma	5,884 (59.06%)	3,446 (58.57%)		
Squamous cell carcinoma	384 (3.85%)	32 (8.33%)		
Non-small cell lung cancer	3,695 (37.09%)	1,110 (30.04%)		
Specimen type			21.205	0
Liquid-based cytological specimens	8,029 (80.59%)	3,788 (47.18%)		
FFPE specimens	1,934 (19.41%)	800 (41.37%)		
Precipitation diameter*			0.175	0.91622
>5 mm	2,201 (27.41%)	1,045 (47.48%)		
2–5 mm	3,872 (48.23%)	2,045 (47.18)		
<2 mm	1,956 (24.36%)	1,040 (46.83)		

*, only liquid-based cytological specimens (8,029 cases) evaluated precipitation diameter. EGFR, epidermal growth factor receptor.

specimens was 47.18% and FFPE specimens was 41.37%. There was statistical difference between the two specimens (data was shown in *Table 1*).

EGFR mutation rate of different specimens

EGFR mutation rate of AC (60.01%) and NSCLC (30.54%) detected by liquid based cytology specimens were higher than FFPE specimens respectively (60.01% *vs.* 30.54% and 54.15% *vs.* 21.99%, χ^2=13.845, P<0.01 and χ^2=16.026, P<0.01). Mutation rate of SCC detected by liquid based cytology specimens was 7.58%, a little lower than FFPE specimens, however, there was no statistical difference between the two groups (*Figure 2*).

EGFR mutations sites in different types of specimens

In 8,029 cases detected by liquid based cytology specimens, 1,811 cases were found 19 Del (47.81%) and 1,665 cases (43.95%) were found L858R point mutations. These two EGFR mutation sites were most frequent. Other mutation sites included 85 20-ins cases (2.24%), 81 L861Q cases (2.14%), 47 S768I cases (1.24%), 91 G719X cases (2.4%), and 8 cases (0.21%) which have been found two mutation sites (*Figure 3A*). In 1,934 cases detected by FFPE specimens, we detected 360 19 Del cases (45.00%), 384 L858R cases (48.00%), 20 20-ins cases (2.50%), 16 L861Q cases (2.00%), 6 S768I cases (0.75%), 10 G719X cases (1.25%), and only 4 double mutation cases (0.50%) (*Figure 3B*).

EGFR mutations in different sample types of liquid based cytology

In 6 different sample types, PE has the highest EGFR mutation rate (695/1,109, 62.67%). The second was FNA samples (2,372/4,988, 47.55%). The EGFR mutation rate of bronchial brush and EBUS-TBNA was 38.44% (316/822) and 37.11% (380/1,024). The lowest EGFR mutation rate was sputum (29/86, 33.72%) (χ^2=180.122, P<0.01). Data was shown in *Figure 4*.

Efficacy of TKI therapy in patients with EGFR mutations

Among 3,788 patients with EGFR mutation tested by liquid-based cytological specimens, we followed 107 cases

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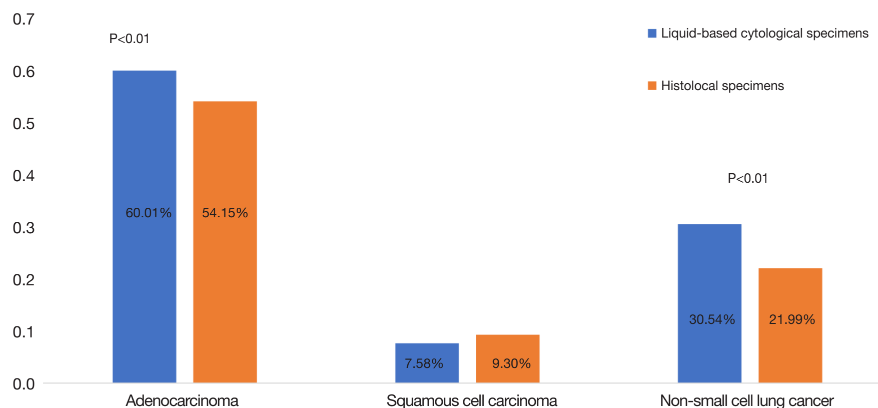

Figure 2 EGFR mutation rate in liquid-based cytology and FFPE specimens. EGFR, epidermal growth factor receptor.

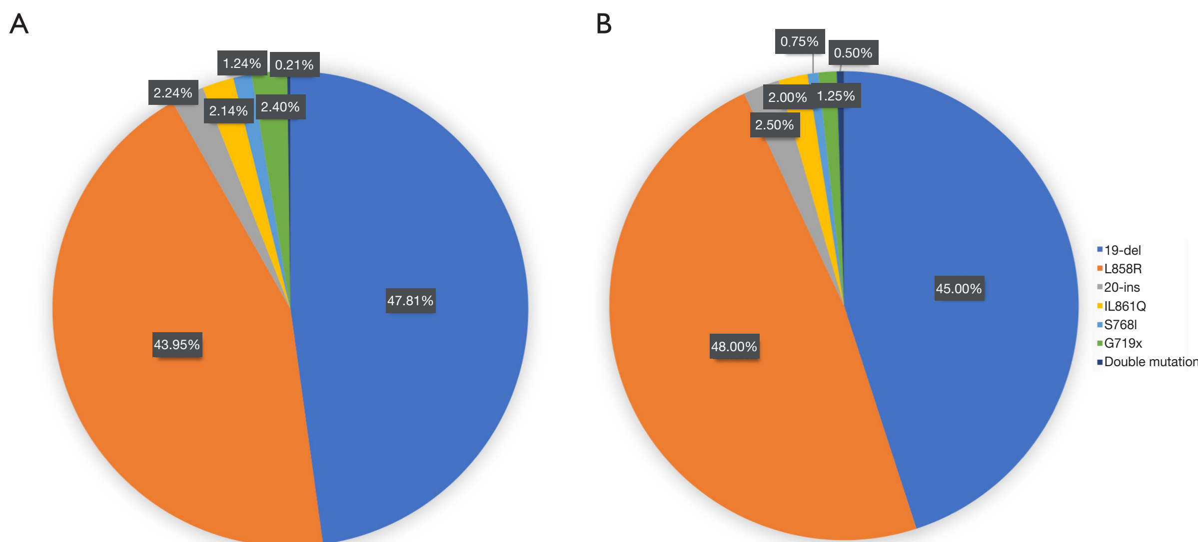

Figure 3 Chart of main EGFR mutation sites distribution. Each mutation site in the figure is shown as a percentage of all tested EGFR mutated cases in all EFGR testing cases. 19-del indicate EGFR absence of 19 exosomes, L858R indicate EGFR 21 exon point mutation, ins 20 indicates EGFR 20 exon insertion, L861Q indicate EGFR 21 exon point mutation, S768I indicate EGFR 20 exon point mutation, G719X indicate EGFR 18 exon point mutation, T790M indicate EGFR 20 exon point mutation, The double mutation indicates that two points of mutations were detected in the same case. (A) The proportion of EGFR mutation detected by residual liquid-based cytology specimens. (B) The proportion of EGFR mutations detected by FFPE specimens. EGFR, epidermal growth factor receptor.

to track the efficacy of patients with positive EGFR testing. Fifty-seven patients (53.27%) achieved partial response (PR) within 1 month, 41 patients (38.32%) achieved stable disease (SD), only 9 patients (8.41%) showed disease progression (PD) in 1 month. The objective response rate (ORR) was 53.27% and disease control rate (DCR) was 91.59%.

Discussion

It's very important for NSCLC patients to detect drive mutation, as it's essential for therapeutic regime and prognosis. In various types of drive mutation, EGFR gene mutation is the most frequent one (10). Accurately screened out EGFR mutations in NSCLC can significantly improve the prognosis of patients. To NSCLC patients

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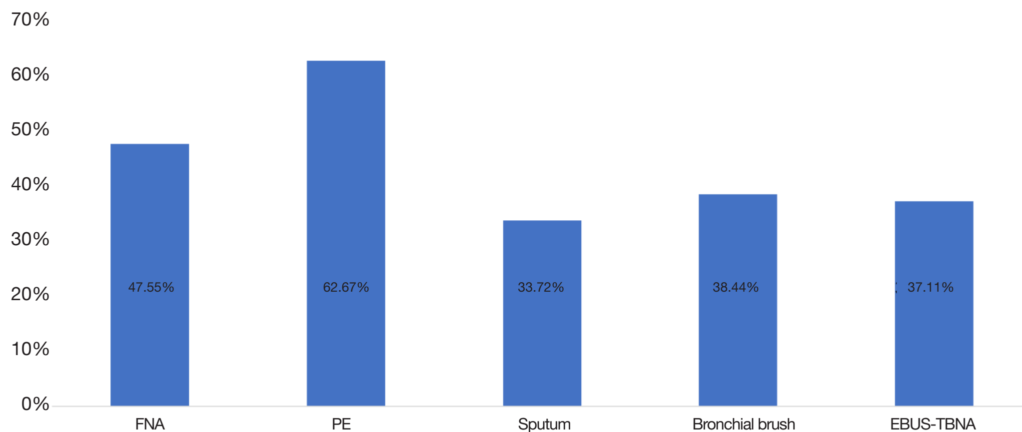

Figure 4 EGFR mutation rate in different types of liquid-based cytological specimens. Data was shown as a proportion of EGFR mutations cases in all EGFR tested cases. EGFR, epidermal growth factor receptor.

without indication of surgery, cytology specimen is the only pathological sample available. Cytology samples can not only provide pathological diagnosis, but also can be used to detect gene mutation in NSCLC patients (11). In addition to liquid based specimens, cell block embedded specimens and cell smear scraping specimens are also used for molecular detection (12,13). However, it is difficult to make a cell block embedded specimen successfully in case of little cells, and cell smear, sometimes as the only pathological slide of patients, will not be filed after scraping. Liquid based cytology is the preferred specimen for molecular detection in Cleveland Clinic in the United States, according to Doxtader *et al.* (14). Therefore, the use of liquid based cytology is particularly important for the detection of gene mutation. In our previous study, we established an evaluation system of EGFR mutation detection by liquid based cytology specimens. Here, we summarized our previous experience regarding EGFR gene mutation based on 8,029 cases of cytology samples and evaluated the mutation frequency of EGFR in NSCLC patients in China.

The EGFR mutation rate of NSCLC patients tested by liquid based cytology samples was quite different in literatures (3,4). There are many associated factors. The most important one is related to the pre-treatment of specimens and the establishment of evaluation before EGFR mutation test. A large number of samples were explored in our laboratory in the early stage, and the evaluation of tumor cell content of liquid-based cytology samples before molecular detection was established, and it was applied to daily work. For the cases with less than 50 tumor cells in the liquid based cytology slide, it is not recommended to use this liquid based cytology sample for EGFR mutation detection. Even if the diameter of centrifugation precipitation is large, there are possibility to have plenty of normal epithelial cell or other components mixed. In this case, the tumor cells account for less, when the EGFR mutation test result is negative, it may be a false negative. It is recommended to sample again or use more sensitive detection methods, such as super arms PCR, to detect molecular mutation. It is also not suitable to test the samples with too small sedimentation diameter which was invisible to the naked eye, because the results also have the possibility of false negative, and also the high cost of gene test brings the patients high economic burden. If there is no other samples obtained or no chance to resample again, EGFR mutation test can be done by this unqualified sample after fully communication with patients and clinicians, however, it is not recommended to carry out test in daily work.

In our study, the EGFR mutation rate in women was significantly higher than that in men. And the EGFR mutation rate was highest in AC and lowest in SCC, which was consistent with the literature (15). Further analysis showed that the EGFR mutation rate in AC and NSCLC tested by liquid based cytology samples was higher than FFPE samples. It has been reported that cytology samples can provide high quality DNA for molecular detection, and the results are consistent with those of FFPE samples including surgical resection samples and biopsy samples (16). Because of the high proportion of tumor cells in cytology specimens, cytology specimens were more suitable

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for molecular detection according to Roy-Chowdhuri *et al.* (17). On the other hand, for liquid based cytology specimens, pathological diagnosis only depends on cell morphology. Poor differentiation NSCLC lacks the typical cell morphology of AC and SCC, and cannot be further defined by immunohistochemistry, resulting in a proportion of poorly differentiated AC, SCC, adenosquamous cell carcinoma and large cell carcinoma in NSCLC group of cytological specimens. Therefore, the EGFR mutation rate was higher in NSCLC group. We believe that EGFR should be tested in NSCLC patients, especially those who depend on cytological diagnosis, so as to avoid missing the opportunity of EGFR TKI treatment.

According to the different sampling methods, the liquid based cytology samples are divided into FNA, PE, sputum, bronchial brush, BALF and EBUS-TBNA samples. Among these samples, we found PE had the highest EGFR mutation rate, reaching 63.35%. There are two reasons for the high EGFR mutation rate in PE. First, AC is the most common pathological type of malignant pleural effusion in lung cancer patients (18). Secondly, a series of recent reports showed that lung cancer has large heterogeneity in tumor (19). For example, the gene characteristics of primary and metastatic lesions will also be different, and the DNA of tumor cells can also be released into PE through necrosis or apoptosis of tumor cells (20). Therefore, PE can detect the presence of gene mutation better than a biopsy sample with limited tumor cells (20). In recent years, many studies have also shown that the detection of driving mutation in PE can provide reliable molecular detection results for patients treated with TKI, and even the supernatant of pleural effusion with negative cytological diagnosis can also be used as reliable liquid biopsy samples (21,22). PE can be collected quickly, minimally invasive and repeatedly. For patients with advanced NSCLC patients with PE, it is recommended to send PE samples for EGFR mutation detection and EGFR TKI treatment monitoring.

For NSCLC patients with EGFR-exon 19 deletions or an exon 21 Leu858Arg mutation, EGFR TKIs can improve response rates, time to progression, and overall survival. Consist with literature (23), we found the ORR was 53.27% and disease control rate (DCR) was 91.59%, only 8.41% patients showed PD in 1month. There are different mechanisms of acquired resistance to EGFR TKIs. T790M mutation, HER2 amplification, MET amplification and small cell lung cancer transformation are among the highest (23).

Since next-generation sequencing hasn't carried out universally, we haven't compared the results of EGFR gene mutation detection with it. In future study, EGFR gene status will be compared between ARMs and next-generation sequencing.

In conclusion, EGFR detection with liquid-based cytological sample is less invasive and easy to obtain samples. When establishing standardized process and evaluation system of samples, it can provide accurate detection results to guide TKI treatment, and it can also monitor drug resistance of NSCLC patients after TKI treatment. It is recommended to promote its application. In addition, EGFR mutation detection is still recommended for patients with NSCLC, especially diagnosed with cytology samples. In all kinds of cytology specimens for the EGFR detection, PE is first choice.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at http://dx.doi.org/10.21037/jtd-20-2750

Data Sharing Statement: Available at http://dx.doi.org/10.21037/jtd-20-2750

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/jtd-20-2750). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Ethical approval for the study was obtained from the ethical committee of Shanghai Pulmonary Hospital (approval number: L20-331Y), and written informed consent was obtained from the patients.

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