RESEARCH ARTICLE

High Feasibility of Liquid-Based Cytological Samples for Detection of EGFR Mutations in Chinese Patients with NSCLC

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Abstract

Background: Activating mutations of epidermal growth factor receptor (EGFR) could predict response to tyrosine kinase inhibitor (TKI) treatment in patients with non-small cell lung cancer (NSCLC). However, the detection of EGFR mutation is frequently challenging in clinical practice for the lack of tumor tissue. The aim of this study was to investigate the feasibility of performing EGFR mutation testing on various types of liquid-based cytology (LBC) samples. Materials and Methods: A total of 434 liquid-based cytology samples were collected from March 2010 and November 2013. Among them, 101 with diagnosis of lung adenocarcinoma had paired surgically resected specimens. The ADx Amplification Refractory Mutation System (ADx-ARMS) was used to determine EGFR mutation status both in LBC and resected samples. Results: All liquid-based cytology samples were adequate for EGFR mutation analysis. The mutation rate was 50.5% in the 434 NSCLC patients with LBC samples and the incidence rates of EGFR mutation were consistent among different specimens. We also detected EGFR positives in 52.5% (53/101) patients with paired histologic specimens. The concordance rate of EGFR mutation between LBC samples and paired histologic specimens was 92.1%. Conclusions: Our results suggest that liquid-based cytology samples are highly reliable for EGFR mutation testing in patients with NSCLC.

Keywords: EGFR mutation liquid - based cytology - lung adenocarcinoma - ADx Amplification Refractory Mutation System

Asian Pac J Cancer Prev, **15 (18)**, 7885-7889

Introduction

Activating mutations in epidermal growth factor receptor (EGFR) gene confer dramatic sensitivity to EGFR TKIs (Rich et al., 2004). Several large randomized phase III studies consistently showed that EGFR-TKIs, such as gefitinib, erlotinib or afatinib achieved significantly higher response rates and longer progression-free survival (PFS) compared to standard chemotherapy and should be regarded as the optimal 1st-line therapy in this population (Han et al., 2012; Inoue et al., 2013; Chen et al., 2013; Wu et al., 2014). However, it is difficult to get enough tumor tissue for genotyping of EGFR in advanced NSCLC patients. Thus, the EGFR detection rate was as low as less than 10% in a national survey in China in 2011. Therefore, identifying other alternate samples to detect EGFR mutation status is urgently needed in the clinical practice.

In clinical practice, cytology samples such as computed tomogram guided fine-needle aspiration cytology (FNA), bronchofiberscopic brushing (BB) or bronchoalveolar lavage (BL), Endobronchial ultrasound guided transbronchial needle aspiration (EBUS-TBNA), Pleural effusion (PE) and Sputum are the main source to get the diagnosis of NSCLC. Conventionally, these cytological samples were preserved in CytoLyt solution to make into liquid-based cytological (LBC) slides. It has been reported that driver genes analysis was successfully performed in conventional smears or cell blocks (Aisner et al., 2013; Sun et al., 2013; Khode et al., 2013). As we know, CytoLyt solution can prevent degradation of tumor cell DNA. Meanwhile, LBC DNA was obtained directly from CytoLyt solution which avoided loss of some tumor cells in the process of conventional smears or cell blocks. Thus, LBC samples might be proper and provide adequate DNA amount for analyzing EGFR mutation status (Malapelle et al., 2012; Dejmek al., 2013).

To investigate the feasibility of detecting EGFR mutation status through LBC samples in clinical practice, we use the methods of ARMS to perform the genotyping analysis through various types of liquid-based cytology (LBC) samples (Pao et al., 2007; Ellison et al., 2010). Besides that, we also compared EGFR mutation status between LBC specimens and paried surgical samples to investigate whether cytological samples could act as an alternate one.

Materials and Methods

Sample Collection

A total of 434 available LBC from primary or metastatic

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lesions were collected for testing EGFR mutation: 260 (59.91%) from primary tumors, including 221 were obtained using computed tomogram guided fine-needle aspiration (CT-FNA), 50 by means of bronchofiberscopic brushing (BB) or bronchoalveolar lavage (BL), 9 sputum, 174 (40.09%) from metastatic lesions, including 113 pleural effusion (PE) and 61 by endobronchial ultrasound guided transbronchial needle aspiration (EBUS-TBNA) of mediastinal lymph nodes. Meanwhile, we retrospectively assessed the EGFR mutation status of 101 primary adenocarcinomas surgically resected with paired LBC samples including 40 FNA, 19 PEs, 24 EBUS-TBNA, 18 BB at our hospital between March 2010 and November 2013.

All of the FNAs were performed using disposable 21- or 22- gauge needles, the part of FNA samples were directly smeared to a glass slide and fixed using ethanol, and the rest were processed using the liquid-based, thin-layer cytology ThinPrep 2000 method. The material was fixed using Cytolyt hemolytic and preservative solution according to the manufacturer's procedure. The FNA smears were stained using hematoxylin and eosin for diagnosis. All original slides were reviewed independently by 2 pathologists. This study was approved by the Ethics Committee of Shanghai Pulmonary Hospital, Tongji University, Shanghai, China and a written informed consent was obtained from each participant before the initiation of any study related procedure.

DNA extract

Genomic DNA was extracted from histologic specimens with formalin-fixed, paraffin-embedded tissue according to manufacturer's protocols by The QIAamp DNA Mini Kit (Qiagen, Germany). After deparaffinization using xylene, the target lesions which represented lung cancer in each tumor block were selected to minimize any non-neoplastic cell contamination. A prolonged (48 hours) proteinase K digestion was performed on all of the formalin-fixed, paraffin-embedded tissues; this long digestion time releases the amplifiable nucleic acids by reversing formalin-induced crosslinks. Genomic DNA of each LBC specimen was obtained directly from CytoLyt solution. The concentration of DNA samples were measured by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, USA).

EGFR mutation analysis

Human EGFR Gene Mutations Fluorescence PCR Diagnostic Kit (Amoy Diagnostics, Xiamen, China) was used to detect exon 19-21 mutations in real-time PCR reactions, which combines the two technologies of ARMS and Bi-loop Probe according to the manufacturer's protocol as described before (Chu et al., 2013). Real-time PCR was performed using the ABI-7500 real-time PCR system. Data were analyzed using Stratagene Mxpro software to detect the major exon mutation in EGFR. Some cases with EGFR 19 or 21 exons mutation were confirmed by direct sequencing. PCR products were purified with Exonuclease I and Shrimp alkaline phosphatase to stop the enzymatic reaction as described previously (Jia et al., 2011). Treated PCR products were sequenced with Big Dye Terminator v3.1 cycle sequencing method on ABI 3130XL Genetic Analyze (Applied Biosystems, Foster City, CA). Frequent mutation sites were analyzed and determined by using Chromas 2.23 and Seqscape v2.5 software packages with the EGFR (accession no. NM 005228.3)

Statistical Analysis

SPSS statistical software (version 17.0) was used to perform statistical analysis. The Chi-square test was used to compare the concordance rate of EGFR mutations between histological specimens and paried LBC samples and the incidence rate of EGFR mutation between different demographic or clinical subgroups. Statistical significance was designated using a 2- tailed p value <0.05.

Table 1. The Baseline Characteristics in the 434 Patients who had LBC Samples and the Paired 101 Patients who had Resected Tissues

Clinical factors	LBC samples (n=434)		*p* value	Tissue samples (n=101)		*p* value
	EGFR positive	EGFR negative		EGFR positive	EGFR negative	
Age	62 (26-86)	61 (29-87)		57.7 (33-75)	54.5 (33-77)	
Gender			<0.001			0.042
Male	111	159		17	25	
Female	108	56		36	23	
Histology type			<0.001			NA
Adeno	203	177		53	48	
Non adeno	16	38		0	0	
Smoking status			<0.001			0.042
Smoker	98	135		17	25	
Never-smoker	121	80		36	23	
ECOG PS			0.585			NA
0-1		199	192		53	48
2	20	23		0	0	
Stage			0.156			0.244
I-IIIA	68	81		50	48	
IIIB-IV	151	135		3	0	
Sample sites			0.493			0.119
Primary site	135	125		49	48	
Metastasis site	84	90		4	0	

Results

Patients characteristics

Total 434 NSCLC patients with LBC samples were enrolled into this study. Among them, 101 had the paired surgically resected specimens, 37.8% (164/434) were female, 87.6% (380/434) cases was diagnosed lung adenocarcinoma and 44.0% (191/434) were never-smokers. EGFR mutation frequency was significantly higher in female (p<0.001), adenocarcinoma (p<0.001) and never-smokers (p<0.001). The clinicopathologic characteristics of 434 patients was seen in Table 1.

Evaluation of DNA concentration of LBC specimens

DNA concentration was evaluated for the quality of tumor cells available for EGFR mutation analysis from LBC specimens. The concentration of DNA extracted from LBC specimens ranged from 10.0 ng/μl to 935.3 ng/μl. Each 25 μl reaction specimen contained 2 μl of genomic DNA to detect mutations in real-time PCR reactions (less than the recommended 20 ng per reaction).

Table 2. The Incidence of EGFR Mutation in different LBC Samples

	The incidence of EGFR mutation
LBC specimens	219/434 (50.46%)
Fine Needle	115/221 (52.04%)
Pleural effusion	55/113 (48.67%)
EBUS-TBNA	29/61 (47.54%)
BL or BB	15/30 (50.00%)
Sputum	5/9 (55.56%)
LBC from primary tumor	135/260 (51.92%)
LBC from metastatic lesions	84/174 (48.28%)

Abbreviations: LBC: liquid-based cytology, BB or BL: bronchofiberscopic brushing (BB) or bronchoalveolar lavage(BL); EBUS-TBNA: Endobronchial ultrasound guided transbronchial fine-needle aspiration

Results of EGFR exon 19 and 21 mutations from LBC and surgical specimens by ADx-ARMS

Types of LBC samples yielded adequate results for EGFR mutation sequences. Using the EGFR Mutations Diagnostic kit, 434 LBC samples were detected. The overall rate of EGFR mutations occurred 50.46% in LBC specimens. The rate of EGFR mutation from different types of LBC was showed similarly, 115/221 (52.04%) in CT-FNA, 15/30 (50.00%) in BB or BL, 5/9 (55.56%) in sputum from primary tumor, 55/113 (48.67%) in PE and 29/61 (47.54%) in EBUS-TBNA from metastatic lesions, respectively (Table 2). EGFR mutations by ADx-ARMS analysis are shown (Figure 1). Some cases were confirmed by direct sequencing (Figure 2).

Concordance rate of EGFR mutations between 101 LBC samples and paired histologic specimens

The overall concordance rate in EGFR mutation status between histologic specimens and paired LBC specimens was 92.08 % (93/101). EGFR mutations were observed in 53 of total 101 surgial specimens (52.48%) and 51 of 101 paired LBC samples (50.5%) The FNA specimens

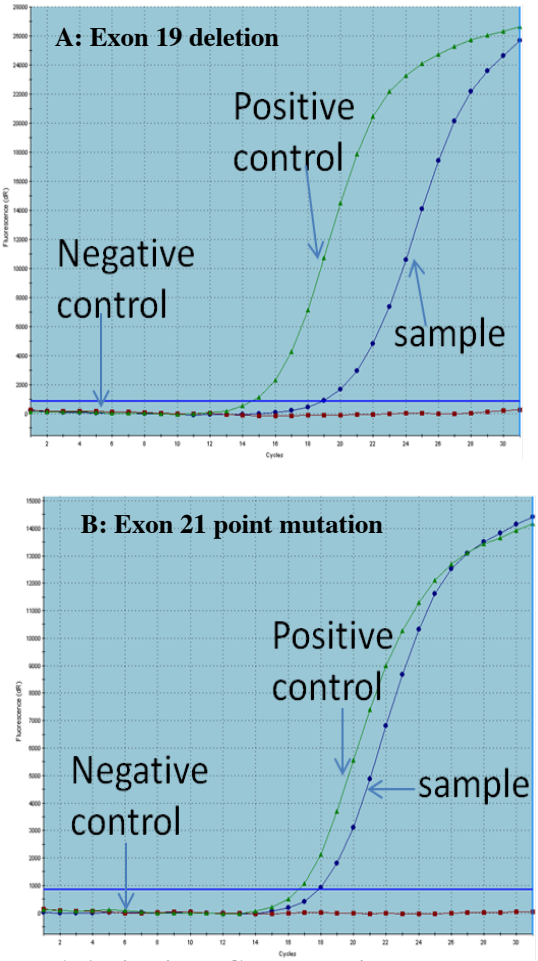

Figure 1. Activating EGFR Mutation Detected by the Method of Scorpion ARMs

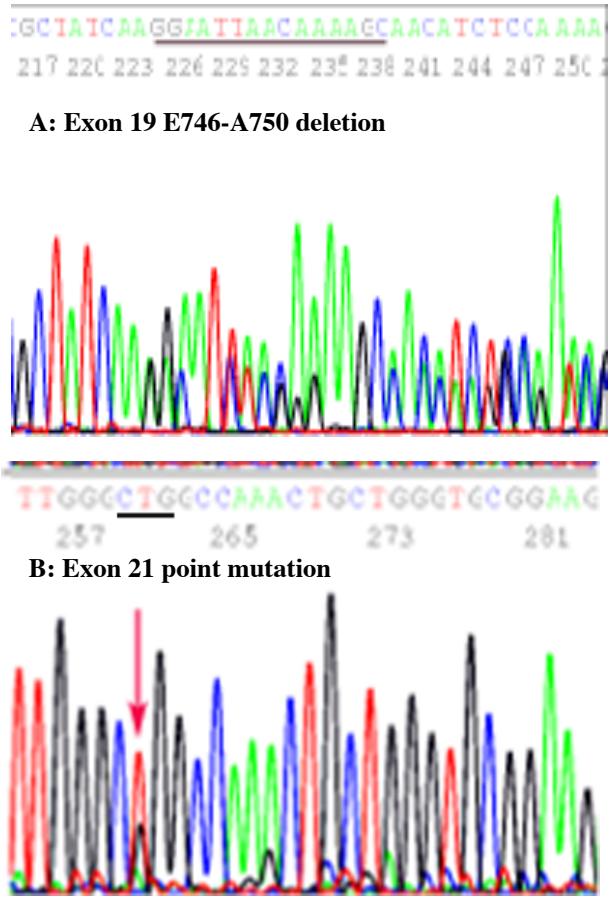

Figure 2. Activating EGFR Mutation Validation by the Method of Direct Sequencing

Table 3. Comparison the Rate of EGFR Mutation Status in 101 Paired Samples

EGFR mutation	pEGFR mut+	pEGFR mut-	Total
cEGFR mut+	48	3	51
cEGFR mut-	5	45	50
Total	53	48	101

P: pathological; C: cytological

exhibited 100% concordance with corresponding histological specimens. EGFR mutation status was discordant in 8 cases including 3 PE, 4 EBUS-TBNA and 1 BB sample (Table 3).

Discussion

In the current study, we demonstrated the feasibility of LBC samples as an alternative specimen for the detection of EGFR mutations. Our study showed that EGFR mutation rate was 50.46% in the 434 NSCLC patients with LBC samples, also the EGFR mutation rates were consistent among different specimens. Furthermore, we compared the incidence of EGFR mutation in 101 paired samples and found a high concordance rate of 92.08% in the EGFR mutation status between histological specimens and types of LBC specimens of lung adenocarcinomas.

EGFR-TKI has showed dramatic effects in advanced NSCLC patients who harboring activating EGFR mutations (Ladanyi et al., 2008; Sholl et al., 2010; Alimujiang et al., 2013), while its effect was limited in patients with EGFR wild type (Lynch et al., 2004). Thus, EGFR mutation testing is critical to guide the use of EGFR-TKI in clinical practice. However, it is still challenging to obtain sufficient tissue to perform molecular analysis in patients with advanced NSCLC. LBC are the most frequent used method in routine clinical practice for the cytological diagnosis and could collect nearly all kinds of cytological samples (Wallace et al., 2007; Wu et al., 2009; Hansen et al., 2011). Besides that, CytoLyt solution contained nucleated cell fixing agents to prevent autocytolysis and DNA or RNA degradation of tumor cells, which will reserve the amount of tumor as more as possible and will be helpful to undergo molecular detection including EGFR mutation. Our results further strengthen this concern. The incidence of EGFR mutation was 50.46% in the enrolled patients who had LBC sample and were consistent in different kinds of cytological specimens, which were numerically higher or similar to the previous reports which used tissue or cytological samples, indicating that LBC sample was feasible to performed EGFR mutation. More importantly, we also found 5 EGFR mutation positive in 9 sputum samples, which suggest that sputum samples, a specimen obtained non-invasively, was also useful to perform the EGFR mutation analysis and need further large scale trial to validation.

Our study further compared the incidence of EGFR mutation in the 101 paired LBC and resected samples. We found a high concordance rate of 92.08% in the EGFR mutation status, which further confirmed that LBC samples could act as an alternative specimen for the detection of EGFR mutations. While we also found that 7.92% (8/101) could just detect activated EGFR mutation in either LBC or resected samples. Recently, the intra-tumor and inter-tumor heterogeneity of EGFR mutation within individuals has been reported (Han et al., 2011; Wang et al., 2014), which means that mutation-positive cells and mutation-negative cells may co-exist. However, a recent other study demonstrated that heterogeneity in the distribution of EGFR mutations is extremely rare in lung adenocarcinomas, which may give rise to the different molecular profiles observed by different studies (Yatabe et al., 2011). In our study, the rate of EGFR mutation of LBC specimens from primary tumors (51.92%) was slightly higher than that from metastatic lesions (48.28%). Therefore, we speculate that the low rate of EGFR mutation from metastatic lesions especially in EBUS-TBNA may be caused not only by heterogeneity in the distribution of EGFR mutations but also by the low quality and quantity of the materials. In clinical practice, it's critical to prevent tumor cells DNA degradation before performing EGFR mutation test and sensitive methods such as next-generation sequencing will be applied in the near future.

We must mention that we have several limitations. First, the nature of retrospective study will induce the collection bias. Second, the cytological samples varies, thus the tumor cell numbers, tumor percentages or DNA concentrations differs, which might affect the sensitivity of EGFR mutation detection (Pang et al., 2012; Khode et al., 2013; Jing et al., 2013). Third, not all of paired samples were collected simultaneously at a same location, the phenomenon of heterogeneity could has an impact on the detection result of EGFR mutation.

In summary, we successfully detected EGFR mutation in 50.46% of LBC samples in patients with NSCLC. Besides that, we compared the EGFR mutation status between histological specimens and LBC samples from patients with lung adenocarcinoma and showed a high concordance rate of 92.08%. Taken together, our study suggested that LBC specimens could be used to detect the EGFR mutation for guiding the individual therapy in patients with NSCLC.

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