

## Suitability of Surgical Tumor Tissues, Biopsy, or Cytology Samples for Epidermal Growth Factor Receptor Mutation Testing in Non–Small Cell Lung Carcinoma Based on Chinese Population<sup>1,2</sup>

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### Abstract

**BACKGROUND:** Epidermal growth factor receptor (EGFR) mutation status is crucial in treatment selection for non–small cell lung cancer (NSCLC) patients; however, the detection materials' availability remains challenging in clinical practice. In this study, we collected surgical resection tissues, lymph node biopsy, and cytological samples for EGFR mutation testing and investigated the associations between gene mutation and clinical characteristics. **METHODS:** Two hundred and seventy-six NSCLC adenocarcinoma specimens were collected, and highly sensitive amplification refractory mutation system method was implemented for EGFR mutation detection, with clinicopathologic characteristics involved in the final analysis. **RESULTS:** In the total of 276 samples, 96% (265/276) of tumors obtained evaluable EGFR mutation status, the frequency of mutation was 55.8% (148/265) in all specimens, and three different type samples shared a comparable successful testing rate: 97.4% (38/39) in surgical tumor tissues, 100% (108/108) in lymph node biopsy samples, and 92.2% (119/129) in cytological samples. EGFR mutation was significantly associated with sex, smoking history, lymph node metastasis status (N stage), primary tumor size, testing tissues origin, and sample type ( $P < .05$ ). Multivariate analysis reconfirmed that smoking history and primary tumor size shared significant correlation with EGFR mutation after adjustment. **CONCLUSIONS:** Both lymph node biopsy and cytological samples were suitable surrogates for EGFR mutation detection in NSCLC compared with tumor tissues, gene status should be detected widely considering the high EGFR mutation rate, and nonsmoking history together with smaller primary tumor size was an independent indicator of EGFR mutation status.

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### Introduction

Lung cancer causes the majority of cancer-related deaths all over the world, of which non–small cell lung cancer (NSCLC) comprises nearly 80% to 85% cases [1]; moreover, approximately 75% of patients presented locally advanced or distant metastasis when diagnosed [2]. Fortunately, with novel biological agents emerging for targeted therapy in cancer treatment, better response and longer survival were observed in many clinical trials [3–5]. These small molecular tyrosine kinase inhibitors (TKIs), such as gefitinib and icotinib, both appeared to have great advantages when compared with chemotherapy for first-line treatment in epidermal growth factor receptor (EGFR) mutant NSCLC patients. Furthermore, detecting EGFR status before TKIs usage as first-line therapy has been widely accepted [6,7].

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<sup>2</sup>Authors' contributions: X.H.H. participated in study design, collected data, carried out data analysis, and drafted the manuscript. Z.S.Z. and D.W. carried out the mutation analysis. Y.C.S. and S.W. performed the statistical analysis and participated in manuscript draft. L.W., Y.T.L., S. Y., and X.S.H. participated in samples collection. Y.F. participated in data collection. Y.S. participated in study design. Y.K.S. conceived the study, design and coordination the study, and draft the manuscript. All authors read and approved the final manuscript.

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EGFR mutation testing in clinical practice has been improved tremendously during the past decade; however, samples' availabilities remain challenging. Generally, tumor tissues are optimal for detecting based on sufficient tumor cells and genome DNA. While nearly 70% of lung cancer patients were diagnosed using biopsy or cytology specimens because of the unavailability of surgical tumor tissues in unresectable and advanced diseases [8,9]. Actually, sufficient diagnostic materials acquisition remains a problem in all populations; therefore, the necessity of diagnosis with small biopsy materials and cytological samples appears more and more important in clinical application. Current data confirm the cytology testing for EGFR mutation, with a promising concordance rate between tissues and cytological samples [10–13], which indicates that small specimens would play as appropriate surrogates in EGFR detection. As gene mutation testing methods increase dramatically, such as the second- or third-generation deep sequencing, Sanger sequencing has been replaced to some extent because of its limitations that include low sensitivity and longer time consumed [14,15], although it is still recognized as the “gold standard” in gene detection. Amplification refractory mutation system (ARMS) is a popular targeted real-time polymerase chain reaction (PCR)-based method for gene mutation detection, with a higher sensitivity, more convenient manipulation, and less turnaround time, which would be proper for EGFR testing especially in insufficient samples like lymph node biopsy and cytology specimens.

Nowadays, tumor tissues, biopsy, and cytological samples are the most common diagnostic materials for clinical testing, and EGFR mutation status appears to have a pivotal role in selecting patients who are most likely to derive benefits from TKI therapy. However, the discordance between EGFR mutation tests for several factors such as variation in tumor cell content and sample size differences remains a challenge [16,17]. In this study, we collected 276 NSCLC adenocarcinoma samples, and the EGFR mutation status was detected by ARMS. We aimed to investigate the EGFR mutation prevalence in different sample types; moreover, associations between gene mutations and clinicopathologic characteristics together with different testing results among these three samples types were analyzed.

## Methods

### *Patients and Samples*

From December 2012 to November 2013, a total of 276 NSCLC adenocarcinoma patients were enrolled in this study with available testing materials. Eligible patients had pathologically confirmed NSCLC adenocarcinoma according to American Joint Committee on Cancer seventh edition criteria and available tumor samples for gene mutation detection. All patients should be over 18 years old, and written informed consent was obtained from each patient before the study. The procedure was approved and supervised by the Institutional Review Board (IRB) of the Cancer Institute/Hospital of Chinese Academy of Medical Sciences and Peking Union Medical College. Smoking is defined as at least one cigarette per day or occasionally for at least 1 year, regardless of past or current status, and patients who had never smoked cigarettes during their lifetime were recognized as never-smokers.

### *DNA Extraction and Mutation Analysis*

Hematoxylin and eosin staining and histologic analysis were used to identify the representative malignant cells in each specimen by two independent pathologists before experiments. Tissue or cell blocks were cut into 5- $\mu$ m sections for formalin-fixed, paraffin-embedded samples, and DNA extraction was performed using the QIAamp

DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA was dissolved in 50  $\mu$ l of deionized water and stored at  $-80^{\circ}\text{C}$  until use. DNA concentration was measured with a NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, USA) by detecting optical absorbance at 260 nm. Then the EGFR RGQ PCR Kit (Qiagen) was used for EGFR mutation detection with the ARMS/Scorpion assay, which allows testing of 29 known mutations for EGFR. PCR results were collected and analyzed according to the manufacturer's protocols.

### *Statistical Analysis*

Statistical analysis was carried out by the SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL). Associations between clinicopathologic characteristics and EGFR status in all samples types were evaluated by the chi-square or Fisher exact tests, and only variables with statistical significance were subjected to final logistic regression analysis, using a backward stepwise (likelihood ratio) method with odds ratio (OR) calculated. The two-side significant level was set at  $P < .05$  through the whole analysis process.

## Results

### *Patients*

We have enrolled 276 patients (128 male and 148 female with a mean age of 56 years) in this study, of which 39 (14.1%) samples were surgical resection tumor tissues, 108 (39.1%) were derived from lymph node biopsy, and 129 (46.8%) cases were cytological samples (98 needle aspiration biopsy, 29 transbronchial endoscopic biopsy, and 2 pleural effusion samples). The patients' demographic and clinicopathologic data are presented in Table 1.

### *EGFR Mutation Assessment*

The EGFR mutation was detected successfully in 265 (96%, 265/276) samples; 148 (55.8%, 148/265) harbored an EGFR mutation, of which 68 (25.7%, 68/265) were deletion in exon 19 and 70 (26.4%, 70/265) were L858R in exon 21. T790M mutation was found in three samples; moreover, two of these coexisted with an L858R mutation. Other mutant types included G719X in exon 18, L861Q in exon 21, or combined mutation deletion (exon 19) with L858R. The spectrum of these mutations was shown in Table 2. All lymph node biopsy samples were detected successfully (108/108, 100%), whereas the test success rates of tumor tissue samples (38/39, 97.4%) and cytological samples (119/129, 92.2%) were somewhat lower. These failed 11 samples included 1 surgical resection tumor tissue and 10 cytological specimens that contained 8 transthoracic needle biopsy samples and 2 transbronchial endoscopic biopsy samples. Before we performed the EGFR mutation detection, strict quality control included DNA concentration, and A260/A280 absorbance ratio was calculated. Nearly all of these samples' DNA concentration was lower than 6.0 ng/ $\mu$ l (only one sample's DNA concentration was 10.0 ng/ $\mu$ l), and most of the absorbance ratio appeared aberrant (seven samples with an A260/A280 absorbance ratio over 2.4 and one sample was lower than 1.7). These 11 samples did not pass the Qiagen kit positive control; therefore, mutation detection was not performed. The mutant rates for detected samples were 63.2% (24/38) in tumor tissues, 46.3% (50/108) in lymph node biopsy samples, and 62.2% (74/119) in cytological samples, respectively. And the mutation rates were significantly different in these three type samples ( $P = .034$ , Table 1).

**Table 1.** Characteristics of 265NSCLC Patients and Association of EGFR Mutations with Clinicopathologic Parameters

Characteristics	Total No.	Wild Type		Mutation	P
		No.	No.		
Sex					
Male	120	69	51		<.0001
Female	145	48	97		
Age, years					
≥56	143	64	79		.572
<56	122	53	69		
Smoking history					
Ever	101	65	36		<.0001
Never	163	52	111		
Missing	1				
Initial diagnosis					
Locally advanced	89	46	43		.116
Distant metastasis	176	72	104		
Tumor stage					
IIIa/IIIb	50	28	22		.118
IV	207	84	123		
Other *	5	1	4		
Missing	3				
T stage					.068
T1	46	13	33		
T2	112	50	62		
T3	21	13	8		
T4	83	39	44		
Missing	3				
N stage					.040
N0	40	17	23		
N1	12	5	7		
N2	61	18	43		
N3	149	76	73		
Missing	3				
M stage					.089
M0	55	29	26		
M1	207	84	123		
Missing	3				
Primary tumor size					
<3 cm	93	30	63		<.0001
>3 to <5 cm	112	40	72		
>5 cm	60	41	19		
Tissue origin					
Lung	130	47	83		.028
Lymph node	121	64	57		
Other †	14	6	8		
Sample type					
Surgical resection tissue	38	14	24		.034
Lymph node biopsy	108	58	50		
Cytology samples	119	45	74		

\* Including four stage II and one stage I patients.

† Including nine pleurae, four bones, and one neck lump.

**EGFR Mutation Analysis**

EGFR mutation appeared more frequently in female than male samples (66.9% vs 42.5%), and never-smoking patients shared a higher mutation status (68.1% vs 35.6%). Although tumor stage did not share a significant difference, the lymph node metastasis status (N stage) showed a different EGFR mutation rate. Besides, with tumor size increased, the EGFR mutation frequency declined. Samples that originated from lung had a higher EGFR mutation rate than lymph nodes, bones, and pleura ( $P < .05$ , Table 1). Multiple logistic regression analysis identified that smoking history ( $P < .0001$ , OR = 0.289, 95% CI 0.157 to 0.533) and primary tumor size ( $P = .001$ , OR = 0.492, 95% CI 0.329 to 0.736) were still significantly associated with EGFR mutation status. We did not observe any other significant association between EGFR mutation and clinicopathologic characteristics (Table 3).

**Table 2.** Summary of the EGFR Mutations' Distribution

	No.	%
Patients with evaluated test	265	100
Patients with EGFR mutation	148	55.8
Single mutation	141	53.2
Deletion, exon 19	68	25.7
L858R	70	26.4
T790M	1	0.37
G719X	1	0.37
L861Q	1	0.37
Combined mutations	7	2.6
L858R, T790M	2	0.74
L858R, L861Q	1	0.37
L858R, S768I	1	0.37
L858R, Deletion	1	0.37
G719X, Deletion	1	0.37
L861Q, S768I	1	0.37
Patients without EGFR mutation	117	44.2

**Discussion**

In this study, we performed EGFR mutation detection in three different types of samples by ARMS, and the results showed that all types of specimens obtained comparable testing success rates (all over 90%). Considering that the acquisition of sufficient materials for diagnosis and molecular detection in advanced NSCLC patients was difficult sometimes, it seemed inevitable that small specimens such as biopsy and cytological samples will be tested for EGFR status. Previous studies have indicated that cytology samples were suitable in EGFR mutation detection [11–13]. Herein we collected needle aspiration biopsy, transbronchial endoscopic biopsy, and pleural effusion samples to confirm the application of these valid surrogates, which was also validated by a recent study [18]. EGFR mutation rates varied significantly in different type of samples. Surgical resection tissues (63.2%) and cytological samples (62.2%) showed a higher mutation rate than lymph node biopsy specimens (46.3%), which was consistent with a recent study [19], although they had a lower EGFR mutation frequency in all samples. The tendency of EGFR mutant status was consistent with previous studies. Female and never-smoking patients shared a higher EGFR mutation frequency [20,21]. We also found that the primary tumor size was significantly associated with EGFR mutation. Smaller tumors indicated higher EGFR mutants. The previous computed tomography scan may provide clues about gene mutation status; however, because the sample size was relatively small in our study, the result should be validated in further studies. Moreover, testing materials that originated from lung had more EGFR mutations compared with lymph node or other origins such as pleurae, bones, and neck lump (EGFR mutation frequency was 63.8%, 47.1%, and 57.1%, respectively;  $P = .028$ ; Table 1). The tumor stage did not share significant association with EGFR mutation in the current study, and only lymph node metastasis status (N stage) showed a different EGFR mutation rate, whereas another study reported that patients with stage IV tumor would be more likely to harbor EGFR mutants ( $P = .016$ ) [22]. We thought this difference may have been impacted by different pathological sample type and region alternations. Future investigations would provide more knowledge in this controversial issue. The multiple logistic regression indicated that never-smoking history and smaller primary tumor size appeared as independent factors in EGFR mutation status, whereas the tissue origin and other factors did not share this. Moreover, because tissue origin obtained a statistical correlation trend with EGFR mutation, although the  $P$  value was

**Table 3.** Multivariate Logistic Regression in NSCLC Patients between EGFR Mutation and Clinicopathologic Parameters

Characteristics	EGFR Mutation	
	Adjusted OR (95% CI)	LRT <i>P</i> value
Sex	0.671 (0.310-1.452)	.311
Smoking history	0.289 (0.157-0.533)	<.0001
N Stage	0.920 (0.682-1.242)	.586
Primary tumor size	0.492 (0.329-0.736)	.001
Tissue origin	0.643 (0.387-1.068)	.088
Sample type	1.061 (0.672-1.678)	.798

LRT: likelihood ratio test; 95% CI: 95% confidence interval.

.088 in this multivariate analysis, we believe that a study with a larger sample size in the future would draw a clearer conclusion.

The EGFR mutation frequency varies between ethnicities. Generally, the Asian population appeared to have more EGFR mutants than the Western population [22–24]. In our study, the incidence of EGFR mutation was 55.8%, which was similar to a recent published study (A prospective, molecular epidemiology study of EGFR mutations in Asian patients with advanced non–small-cell lung cancer of adenocarcinoma histology (PIONEER)) in Asian populations (51.4%) [22]. Although disparities exist in different populations, the EGFR-TKIs efficacy was demonstrated for both races [5,25]. Furthermore, regardless of sex, ethnicity, smoking history, or other clinical risk factors, all patients who are selected for EGFR inhibitors therapy should receive molecular detection, which was recommended by three societies in a recently published guideline [26]. Meanwhile, we have participated in creating the Diagnosis and Treatment Guideline of Chinese Patients with EGFR Mutation and ALK Fusion Gene-Positive Non–Small Cell Lung Cancer (2013 Version) based on Chinese characteristics [27]. However, most NSCLC patients usually suffered from advanced stage when diagnosed. Samples for detection were available only in small sizes, such as biopsy and cytological specimens. In the present study, we confirmed the substitution of biopsy and cytology samples for EGFR test, which was consistent with previous studies [28,29,10]. With the limitation of insufficient DNA in small size samples, a more sensitive method was used in this study.

However, 11 samples did not contribute to the final test, the poor quality of specimens with lack of tumor cells indicated insufficient DNA extraction, and we failed to detect the EGFR mutation status even when using a highly sensitive testing method. These results implied that cytological samples were more likely to undergo detection failure for several reasons such as the size of needle and forceps and the number of biopsies [30,31]. More communications between clinicians and pathologists would reduce the failure and detection turnaround time, as clinicians would endeavor to obtain sufficient materials for diagnosis and molecular analysis. Meanwhile, additional feedbacks would also help provide the rational arrangement of testing procedure and make maximum use of limited samples [8,9,32,33].

Generally, tumor samples for molecular detection vary with different intentions. Fresh frozen samples would be a priority when gene mutation detection was planned prospectively, whereas tissue storage for a long time was required in most cases. To decrease the susceptibility of DNA degradation and ensure the accuracy of testing results in cut sections, the choice of fixative was pretty important. In particular, tumor samples fixed for 8 to 24 hours in neutral-buffered formalin (10%) is preferred, the fixation time could not surpass this range in avoiding under- or overfixation, and Bouin or mercury-containing fixatives should be

excluded [34]. Tumor cells enrichment was required for better quality or more sufficient quantity in formalin-fixed, paraffin-embedded samples, increasing the reliability of testing results. EGFR mutation could be tested in different type specimens, such as surgical resection, open biopsy, endoscopy, transbronchial endoscopic biopsy, and others [11]. In general, larger samples were preferred for the greater amount of malignant content, although small biopsy or cytology specimens were also appropriate for EGFR detection, particularly if the cell blocks were available [35]. In addition, because of the possible contamination of extraction or detection kits, together with the operating procedure were both unpredictable factors, stricter quality control was extremely necessary before and during the detection, samples preparation, testing perform. Time consuming should be taken into consideration together, for the results would guide the selection of treatment and patients' welfare [11,16].

Limitations in the present study included the relatively small study sample size, which would not draw confirmed conclusions in some issues, and no other detection methods for ensuring the testing results to decrease the possible false-positive cases. Besides, because different types of cytology specimens may provide different yields of molecular analysis based on sampling technique used, then we should realize the difference during clinical practice [36]. Furthermore, clinical treatment response and outcomes were not available at present; this information would bring us a step closer to personalized medicine.

In conclusion, lymph node biopsy and cytological specimens were suitable surrogates for EGFR mutation detection in NSCLC. The high EGFR mutation frequency in Chinese NSCLC adenocarcinoma suggested the necessity for testing in pretreatment patients, and never smoking or smaller primary tumor size would provide available information for EGFR mutation status prediction. Finally, emphasis on strict quality control pre- and posttesting was paramount in considering the consequent treatment decision for patients.

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