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Original Article

Accuracy and clinical influence of plasma EGFR mutation detection in management of advanced lung adenocarcinoma

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Abstract: This study aimed to investigate the accuracy of plasma EGFR status detecting according to tissue EGFR status, and further explore the correlation of plasma EGFR status with clinicopathological features and progression-free survival (PFS) in patients with advanced lung adenocarcinoma. 157 patients with advanced lung adenocarcinoma were recruited. Paired tissue and plasma samples were collected before any prior treatments, EGFR gene mutation detection was performed using the amplification refractory mutation system (ARMS) method. EGFR mutations were detected in 81 tissue samples and the mutation rate was 51.6%, while in 50 plasma samples and the mutation rate was 31.8%. The overall concordance rate of EGFR mutations between tissue and plasma samples was 76.4%. And the sensitivity, specificity, positive predictive value and negative predictive value of the EGFR detection using plasma samples were 94.0%, 68.2%, 58.0 and 96.1% respectively. Patients with no-smoking (P = 0.039) and more number of metastatic sites (P = 0.012) presented a higher EGFR mutation frequency in plasma, but no association of plasma EGFR mutation with other clinicopathological features and PFS by targeted therapy was discovered. This study revealed that plasma EGFR mutation detection might be regarded as a good alternative biomarker for lung adenocarcinoma management, especially for patients who were not tolerant with obtainment of tissue samples, but further prognostic value needs to be investigated.

Keywords: Non-small-cell lung cancer, EGFR, plasma, ARMS

Introduction

Lung cancer, as one of malignant tumors with the highest incidence and mortality, is a serious threat to human health worldwide [1]. The 2015 epidemiological data disclosed that about 1.8 million people worldwide were diagnosed with lung cancer in 2012, which accounts for 13% of all new cancer patients; roughly 1.6 million patients died from lung cancer in 2012, corresponding to 20% of the deaths in all cancer patients [2]. Benefit from recent developments in the molecular biology of cancer, the prognosis of cancer has been greatly improved, especially in advanced non-small-cell lung cancer (NSCLC) [3]. By using targeted therapy based on driver genes, the patients' survival time has been dramatically prolonged [4]. The results of the Iressa Pan-Asia clinical study (IPASS) demonstrates that the benefit rate of Iressa in patients with EGFR mutations in tumor tissue is 71.2% which is much greater than that of chemotherapy; however, the benefit rate is lower in patients who do not have any mutations [5]. Accumulating evidences such as CTONG0806 study have illustrated that for lung cancer patients who do not harbor EGFR gene mutations, EGFR-targeted treatment is not recommended due to lacking efficacy of reducing the risk of disease progression or death [6, 7]. And EGFR mutations determination in tumor tissue is highly recommended prior EGFR- tyrosine kinase inhibitor (TIK) therapy according to various guidelines in NSCLC patients [8, 9].

Analysis for EGFR mutations in tumor tissue remains the gold standard for clinical EGFR testing. However, the patients' disease and physical conditions often impede obtaining tumor tissue samples, and sometimes the amount of samples is sufficient for determination. Therefore, in such conditions EGFR muta-

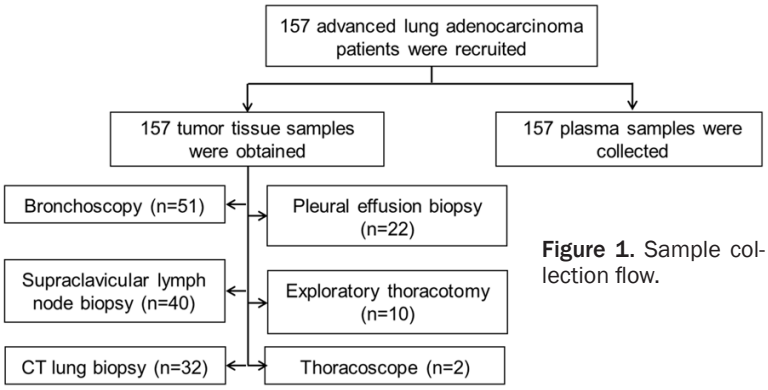

Figure 1. Sample collection flow.

tion testing cannot be carried out effectively, preventing the opportunity for targeted therapies. Several retrospective and prospective studies have shown that when tumor tissues are difficult to obtain, circulating cell-free DNA or tumor DNA (cfDNA/ctDNA) testing can be utilized as an alternative to mutation testing [10-12].

In this study paired tissue and plasma samples were collected from patients with advanced lung adenocarcinoma, and EGFR gene testing was performed using the amplification refractory mutation system (ARMS) method to investigate the accuracy of plasma EGFR status detecting according to tissue EGFR status, and further explore the correlation of plasma EGFR status with clinicopathological features and progression-free survival (PFS).

Patients and methods

Participants

157 patients with advanced lung adenocarcinoma from June 1st, 2012 to October 1st, 2015 in Shanghai Chest Hospital were recruited in this study. Diagnosis was determined by clinical, radiographic and pathological features, and none of these patients received prior radiotherapy, chemotherapy, or other cancer treatments. Among 157 participants, 32 cases were in stage IIIB while the remaining 125 cases were in stage IV. All patients signed informed consent forms to participate in this study and provided permission for the use of their plasma and tumor tissues. This study was approved by the Ethics Committee of Shanghai Chest Hospital.

Samples

Tumor tissues were obtained from all 157 patients, among whom 51 cases were collected by bronchoscopy (TBB, TBLB, EBUS), 32 cases by CT lung biopsy, 40 cases by supraclavicular lymph node biopsy, 22 cases by pleural effusion biopsy, 10 cases by exploratory thoracotomy and 2 cases by thoracoscopy (**Figure 1**). Within a week of obtaining tumor tissues for pathological results, plasma samples (10 mL) were collected with EDTA tubes prior initiation of cancer treatments.

DNA extraction

Tumor tissue DNA extraction: A DNA extraction kit (centrifugal columnar; AmoyDx® FFPE DNA Kit, Amoy Diagnostics, Xiamen, China) for paraffin-embedded tissue samples was used for DNA extraction from tissue samples. DNA samples were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the final concentration was adjusted to 2 ng/μL.

Plasma DNA extraction: A nucleic acid extraction reagent (AmoyDx® Circulating DNA Kit, Amoy Diagnostics, Xiamen, China) was used for DNA extraction from plasma samples. DNA samples were extracted according to the user.

ARMS method for the detection of EGFR mutations

The ABI 7500 fluorescence PCR instrument were used to detect EGFR mutations by ARMS. A human EGFR mutation testing kit (fluorescence PCR assay; Amoy Diagnostics, Xiamen, China) was used to detect EGFR gene mutations. The experiments were performed according to the kit manufacturer's instructions: add 2.7 μL Taq enzyme to 42.3 μL of the DNA sample, mix adequately, and transfer 5 μL of this mixture to the appropriate PCR tube of the 8-tube strip. The following cycling conditions were followed for amplification: 15 cycles of 5 min at 95°C, 25 s at 95°C, 20 s at 64°C, and 20 s at 72°C; 31 cycles of 25 s at 93°C, 35 s at 60°C, and 20 s at 72°C. Fluorescence was

Table 1. Characteristics of lung adenocarcinoma

Parameters	n/% (N=157)
Age (years)	
≤60	95 (60.5%)
>60	62 (39.5%)
Gender	
Man	98 (62.4%)
Female	59 (37.6%)
Smoking status	
No	87 (55.4%)
Yes	70 (44.6%)
Anatomical type	
Central	42 (26.8%)
Peripheral	115 (73.2%)
Stage	
IIIB	32 (20.4%)
IV	125 (79.6%)
Number of metastatic sites	
0	32 (20.4%)
1	79 (50.3%)
2	26 (16.6%)
3	16 (10.2%)
4	4 (2.5%)
ECOG Performance status	
0	2 (1.3%)
1	146 (93.0%)
≥2	9 (5.7%)

Data was presented as count and percentage.

measured at 60°C. Fluorescent signal was collected from FAM and HEX channels. Positive and negative controls were analyzed routinely for lab procedure. The results were analyzed according to the criteria defined by the manufacturer's instructions. Positive results were defined as Ct (sample)-Ct (control) <Ct (cut-off).

Evaluation criteria

Sensitivity, specificity, false negative rate, false positive rate and accuracy rate of plasma EGFR determination were calculated according to tissue EGFR results: Sensitivity (%) = EGFR true positive cases/(EGFR true positive cases + EGFR false negative cases) *100%; specificity (%) = EGFR true negative cases/(EGFR true negative cases + EGFR false positive cases) *100%; False negative rate (%) = EGFR false negative cases/(EGFR true positive cases + EGFR false negative cases) *100%; false positive rate = EGFR false positive cases/(EGFR true negative cases + EGFR false positive cases) *100%; accuracy rate = (EGFR true positive cases + EGFR true negative cases)/(EGFR true positive cases + EGFR false positive cases + EGFR false positive cases + EGFR true negative cases) *100%.

Table 2. EGFR mutation status in tissue and plasma samples

Variable	Case number	Percent of patient (%)
Tissue samples		
Wild	76	48.4
Mutation	81	51.6
19del	42	26.8
L858R	36	22.9
19del + L861Q	1	0.6
G719x + S768I	1	0.6
S768I + L858R	1	0.6
Plasma samples		
Wild	107	68.2
Mutation	50	31.8
19del	25	15.9
L858R	23	14.6
G719x	1	0.6
S768I + L858R	1	0.6

Data was presented as count and percentage.

Statistics

Data was analyzed using the SPSS 20.0 statistical software (Chicago, USA). Data was mainly presented as median value and range, count and percentage. Difference among groups was determined by Chi-square test or Fisher exact test (if appropriate). Kaplan-Meier curve and Log-Rank test were performed to assess the difference of PFS among groups. $P<0.05$ was considered significant.

Results

Characteristics of patients

157 lung adenocarcinoma patients with median age 58.5 years (range 26-77 years) were recruited in this study, among whom 98 (62.4%) were male and 59 (37.6%) were female. 70 (44.6%) patients were smokers and 87 (55.4%) were nonsmokers. According to the anatomical

Table 3. Comparison of EGFR status between tissue and plasma samples

Tissue	Plasma		Total
	EGFR+	EGFR-	
EGFR+	47	34	81
EGFR-	3	73	76
Total	50	107	157

Data was presented as count.

Table 4. Sensitivity, specificity, false negative rate, false positive rate and accuracy rate of plasma EGFR determination

Parameters	
Sensitivity	94.0%
Specificity	68.2%
Accuracy rate (overall concordance rate)	76.4%
Positive predictive value	58.0%
Negative predictive value	96.1%

Data was presented as percentage.

classification, 42 (26.8%) patients had central lung cancer and 115 (73.2%) had peripheral lung cancer. In addition, 32 (20.4%) patients were at stage IIIB while 125 (79.6%) patients at stage IV. Other clinicopathological features were listed in **Table 1**.

EGFR mutations in lung adenocarcinoma

The ARMS method was used to detect EGFR mutations in the tissue and plasma samples collected from 157 patients with lung adenocarcinoma. EGFR mutations were detected in 81 tissue samples and the mutation rate was 51.6% (**Table 2**). Single mutations were detected in 78 samples, in which 42 (51.9%) exhibited the 19del mutation only, and 36 (44.4%) harbored the L858R mutation only. Three samples exhibited double mutations, 1 for 19del + L861Q, 1 for G719x + S768I, and 1 for S768I + L858R, respectively. In the plasma samples, EGFR mutations were detected in 50 samples and the mutation rate was 31.8% (**Table 2**). Single mutations were detected in 49 samples, in which 25 (50%) exhibited the 19del mutation only, 23 (46%) displayed the L858R mutation only, and one showed the G719x mutation. The remaining sample exhibited the S768I + L858R double mutation. The T790m mutation was not detected in any of these samples.

Comparison of EGFR mutations in plasma and tissue samples

As shown in **Table 3**, 47 cases exhibited mutations in both the tissue samples and the paired plasma samples and no mutations were detected in 73 cases. In 34 cases, mutations were detected only in tumor tissue samples and not in the paired plasma samples. In 3 cases, mutations were detected only in plasma samples and not in the paired tumor tissue samples. The overall concordance rate of EGFR mutations between tissue and plasma samples was 76.4%. Taking the test results of the tissue samples as the gold standard, the sensitivity and specificity of the EGFR detection using plasma samples were 94.0% and 68.2% respectively (**Table 4**), and the positive and negative predictive values of plasma samples were 58.0% and 96.1%, respectively (**Table 4**).

Correlation of EGFR mutation in tissue and plasma with clinicopathological features

To analyze the association between clinicopathological characteristics and EGFR mutation status, patients were divided into groups according to their clinical and pathological features. As described in **Table 5**, tissue EGFR mutation was more frequently in younger patients (P = 0.050), female (P = 0.013) and non-smokers (<0.001), no difference were observed between other features and tissue EGFR mutation. As to plasma EGFR mutation, patients with no-smoking (P = 0.039) and more number of metastatic sites (P = 0.012) presented a higher EGFR mutation frequency. And the frequency was numerically increased in central type compared with peripheral type (P = 0.074).

PFS analysis by EGFR status and treatments

As the first-line treatment, targeted therapy (targeted therapy include iressa, tarceva and Icotinib) was administered to 28 patients, chemotherapy to 127 patients, and palliative treatment to 2 patients. According to the EGFR mutation status and first-line treatment strategies, patients were divided into the following three groups: EGFR mutation-positive patients who received targeted therapy, EGFR mutation-positive patients who did not receive targeted therapy, and EGFR mutation-negative patients who did not receive targeted therapy. The medi-

Table 5. Correlation of EGFR mutation by tissue and plasma with the clinicopathological features

Parameters	Tissue EGFR-	Tissue EGFR+	*P*	Plasma EGFR-	Plasma EGFR+	*P*
Age (years)			0.050			0.541
≤60	40 (42.1%)	55 (57.9%)		63 (66.3%)	32 (33.7%)	
>60	36 (58.1%)	26 (41.9%)		44 (71.0%)	18 (29.0%)	
Gender			0.013			0.256
Man	55 (56.1%)	43 (43.9%)		70 (71.4%)	28 (28.6%)	
Female	21 (36.0%)	38 (64.0%)		37 (62.7%)	22 (37.3%)	
Smoking status			<0.001			0.039
No	30 (34.5%)	57 (65.5%)		53 (60.9%)	34 (39.1%)	
Yes	46 (65.7%)	24 (34.3%)		54 (77.1%)	16 (22.9%)	
Anatomical type			0.400			0.074
Central	18 (42.9%)	24 (57.1%)		24 (57.1%)	18 (42.9%)	
Peripheral	58 (50.4%)	57 (49.6%)		83 (72.2%)	32 (27.8%)	
Stage			0.846			0.351
IIIB	15 (46.9%)	17 (53.1%)		24 (77.5%)	8 (22.5%)	
IV	61 (48.8%)	64 (51.2%)		83 (66.4%)	42 (33.6%)	
Number of metastatic sites			0.656			0.012
0	15 (46.9%)	17 (53.1%)		24 (77.5%)	8 (22.5%)	
1	42 (53.2%)	37 (46.8%)		58 (73.4%)	21 (26.6%)	
2	12 (46.2%)	14 (53.8%)		17 (65.4%)	9 (34.6%)	
3	6 (37.5%)	10 (62.5%)		8 (50.0%)	8 (50.0%)	
4	1 (22.5)	3 (77.5%)		0 (0%)	4 (100%)	
ECOG Performance status			0.171			0.852
0	2 (100%)	0 (0%)		1 (50.0%)	1 (50.0%)	
1	68 (41.0%)	78 (49.0%)		100 (68.5%)	46 (31.5%)	
≥2	6 (66.7%)	3 (33.3%)		6 (66.7%)	3 (33.3%)	

Data was presented as count (percentage). Difference was compared by Chi-square test. P<0.05 was considered significant.

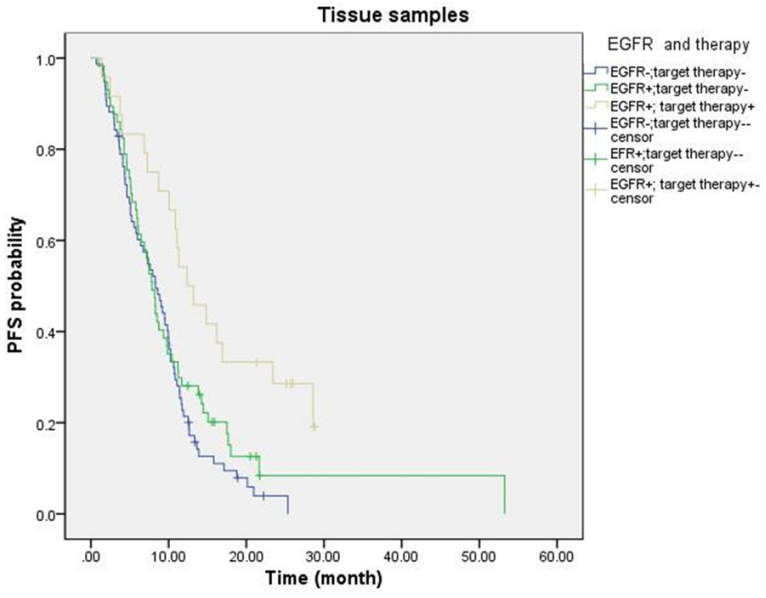

Figure 2. Kaplan-Meier curve analysis of tissue EGFR mutation and target therapy for PFS.

an PFS was compared between the groups. The EGFR mutation testing results of the tumor tissue samples showed that the PFS of EGFR mutation-positive patients who received targeted therapy was significantly longer than that of EGFR mutation-positive and -negative patients who were not treated with targeted therapy. The median PFS of the three groups were 12.4 months, 7.9 months, and 8.3 months, respectively (P<0.001; **Figure 2**). As to plasma sample, EGFR test results of the plasma samples revealed median PFS for the three groups were 10.9 months, 7.9 months,

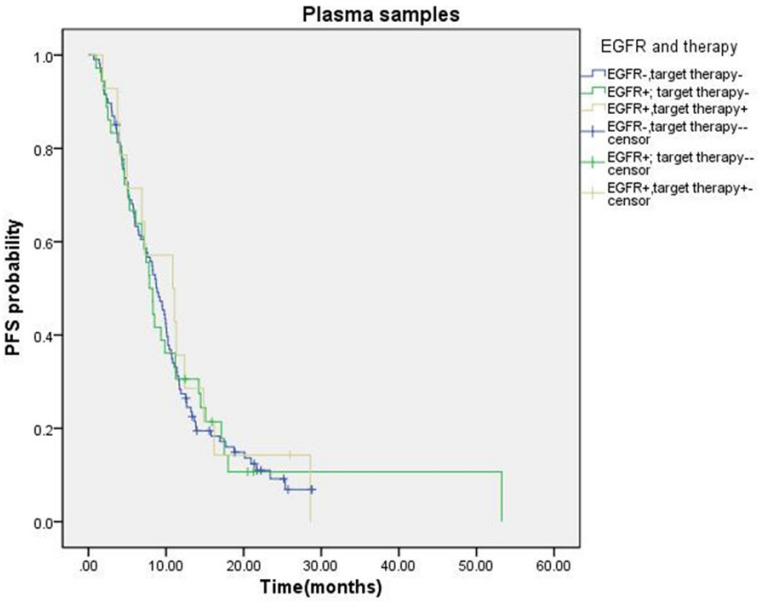

Figure 3. Kaplan-Meier curve analysis of plasma EGFR mutation and target therapy for PFS.

and 8.8 months, respectively, without statistically difference among the three groups (P = 0.725; **Figure 3**).

Discussion

The expression of EGFR is regulated by the oncogene C-erbB-1 (HER-1), which is located on the cell membrane, and it transmits signals to the nucleus primarily through Ras-Raf-MAPK pathway as well as PI3K-PKC-IKK pathway [13]. Aberrant EGFR signaling is widely considered as one of the main causes of various types of tumors [14]. At least 28 different mutations in the EGFR tyrosine kinase domain have been discovered, most of which occur in the exons 18-21 and encode the EGFR tyrosine kinase domain [13]. The four main mutations in NSCLC patients are associated with the sensitivity to the small-molecule TKI, namely the G719A/C mutation (exon 18), an in-frame deletion mutation of four amino acids (Leu-Arg-Glu-Ala) in exon 19, the L858R mutation, and the L861Q mutation (exon 21) [15]. This is possibly due to mutations changing the structure of the EGFR intracellular ATP-binding domain and improving the EGFR binding capacity of TKI [9, 16]. About 20 different deletion mutations of amino acids 747-750 in exon 19 are reported, accounting for about 45% of the mutations. The two most common types of delE746-A750 are 2235_2249del15 and 2236_2250del15, which take up 74% of the deletions in exon 19. Substitution mutations of amino acid 858 in exon 21 account for about 40-45% of the mutations. Mutations in exon 18 (G719S or G719C) make up 5% of the mutations. Insertion mutations in exon 20 account for about 1% of the mutations [14].

In the EGFR tyrosine kinase domain, three types of mutations are associated with tumor drug resistance, including the D761Y mutation (exon 19), T790M mutation, and D770-N77insNPG mutation (exon 20) [17]. In Western countries, the tissue-EGFR mutation rate in NSCLC patients was 10% to 15%, while it was 30% to 40% in Asian populations [18]. And EGFR mutations were more common in young females and nonsmokers [9, 16]. Among the selected Chinese patients with lung adenocarcinoma, the tissue-EGFR mutation rate was about 50% among nonsmokers [5]. In this study, the overall tissue-EGFR mutation rate of patients with lung adenocarcinoma was 51.6%, while it was increased in younger patients (57.9%), female (64.0%) as well as non-smokers (65.5%), which were consistent with previous reports.

Currently, samples used for clinical EGFR testing are mostly surgical tissue samples from tumor sites, biopsy tissues, and cytology samples. However, for a considerable number of patients, tissue samples could not be obtained or the obtained amount is not sufficient for diagnosing molecular subtypes, which prevents the opportunity for targeted treatment. Peripheral blood contains tumor cells, cell-free DNA, RNA, and protein, which all show tumor characteristics to some extent. Additionally, non-invasive and easily obtainable approaches are promising alternatives to taking tumor tissue samples. Blood samples are also known as “liquid biopsy” samples [19, 20]. Currently, ARMS is the most common method for detecting mutations and it is also the EU-approved

method for blood testing during Iressa treatment [21]. In this study, we compared the EGFR test results of tissue and plasma samples using the ARMS method. The concordance rate between tissue and plasma samples was found to be 76.4% (120/157). Taking tissue samples as the gold standard, the sensitivity and specificity of the EGFR detection using plasma samples were 94.0 and 68.2%, respectively. One possible explanation for the discrepancy was tumor heterogeneity: the DNA extracted from these tumor tissue samples harbored EGFR-negative mutations, while the DNA extracted from the plasma samples carried all-round mutations. Another explanation for the discrepancy might be attributed to the cross linking of formalin with DNA during the preparation of paraffin-embedded tumor tissue samples, which induced the fragmentation and degradation of DNA itself. Overall, EGFR detection based on plasma samples revealed a high sensitivity, while a moderate sensitivity. The positive predictive value was 58.0% and the negative predictive value was 96.1%.

The survival analysis showed that the median PFS of EGFR mutation-positive patients who received targeted therapy was significantly longer than that of EGFR mutation-positive and -negative patients who were not treated with targeted therapy by tissue sample examination ($P<0.001$). But the plasma samples showed no significant difference in the median PFS between the three groups, which may be related to the false-negative results of the EGFR detection in plasma samples. In plasma samples, we found that the EGFR detection rate improved with an increase in the number of metastatic organ sites in whole body, suggesting that EGFR detection in plasma tends to be beneficial for patients with advanced metastasis.

In conclusion, this study revealed that plasma EGFR mutation detection might be regarded as a good alternative biomarker for advanced lung adenocarcinoma management, especially for patients who were not tolerant with obtainment of tissue samples, but further prognostic value needs to be investigated.

Disclosure of conflict of interest

None.

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