Peripheral Blood for Epidermal Growth Factor Receptor Mutation Detection in Non-Small Cell Lung Cancer Patients

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Abstract

OBJECTIVE: It is important to analyze and track Epidermal Growth Factor Receptor (EGFR) mutation status for predicting efficacy and monitoring resistance throughout EGFR-tyrosine kinase inhibitors (TKIs) treatment in non-small cell lung cancer (NSCLC) patients. The objective of this study was to determine the feasibility and predictive utility of EGFR mutation detection in peripheral blood. METHODS: Plasma, serum and tumor tissue samples from 164 NSCLC patients were assessed for EGFR mutations using Amplification Refractory Mutation System (ARMS). RESULTS: Compared with matched tumor tissue, the concordance rate of EGFR mutation status in plasma and serum was 73.6% and 66.3%, respectively. ARMS for EGFR mutation detection in blood showed low sensitivity (plasma, 48.2%; serum, 39.6%) but high specificity (plasma, 95.4%; serum, 95.5%). Treated with EGFR-TKIs, patients with EGFR mutations in blood had significantly higher objective response rate (ORR) and insignificantly longer progression-free survival (PFS) than those without mutations (ORR: plasma, 68.4% versus 38.9%, P = 0.037; serum, 75.0% versus 39.5%, P = 0.017; PFS: plasma, 7.9 months versus 6.1 months, P = 0.953; serum, 7.9 months versus 5.7 months, P = 0.889). In patients with mutant tumors, those without EGFR mutations in blood tended to have prolonged PFS than patients with mutations (19.7 months versus 11.0 months, P = 0.102). CONCLUSIONS: EGFR mutations detected in blood may be highly predictive of identical mutations in corresponding tumor, as well as showing correlations with tumor response and survival benefit from EGFR-TKIs. Therefore, blood for EGFR mutation detection may allow NSCLC patients with unavailable or insufficient tumor tissue the opportunity to benefit from personalized treatment. However, due to the high false negative rate in blood samples, analysis for EGFR mutations in tumor tissue remains the gold standard.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide [1]. Non-small cell lung cancer (NSCLC) comprises approximately 85% of all lung cancer cases, of which more than 70% are initially diagnosed with unresectable advanced disease [2,3]. Systemic treatment, including molecular-targeted therapy, plays a central role in the clinical management of NSCLC.

Small-molecule tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, specifically target epidermal growth factor receptor

(EGFR) and generate much optimism in the treatment of NSCLC. *EGFR* mutations have been demonstrated to be the strongest predictive biomarkers for the efficacy of EGFR-TKIs [4–8]. Patients with *EGFR* activating mutations, mainly in-frame deletions in exon 19 (19Del) and L858R substitutions in exon 21, have dramatic tumor responses and favorable survival benefit from EGFR-TKIs [9,10]. However, most responsive patients would eventually experience progressive disease (PD). The secondary T790M mutation in exon 20 accounts for approximately 50% of the mechanism of acquired resistance [11]. Hence, it is of great clinical importance to analyze and track *EGFR* mutation status for predicting efficacy and monitoring resistance throughout EGFR-TKIs treatment in NSCLC patients.

EGFR mutation analysis is recommended in National Comprehensive Cancer Network clinical guidelines for NSCLC. Nevertheless, a national survey shows that only 9.6% of NSCLC patients with stage IIIb or IV disease had *EGFR*-related testing performed in China [12]. Partially because tumor tissue, the optimal DNA source for *EGFR* mutation analysis, is always difficult to obtain. Most NSCLC patients presenting inoperable advanced disease cannot provide surgical samples, whereas biopsy samples may not be sufficient for both pathologic examination and mutation analysis. Besides, many patients refuse repeated biopsy at the time of disease progression. However, peripheral blood of cancer patients frequently contains circulating free DNA (cfDNA) derived from tumor cells, which has been used to detect tumor-specific alterations [13]. Moreover, blood sampling is minimally invasive, readily accessible, relatively repeatable. Thus, using blood for *EGFR* mutation identification and follow-up shows promise.

Amplification Refractory Mutation System (ARMS) has been extensively used in large clinical trials, and has been proved to be a stable, highly sensitive and specific method for *EGFR* mutation detection in tumor tissue. This method was shown to be able to detect mutations in samples containing as little as 1% mutated DNA [4,14–16]. In this study ARMS was used to detect *EGFR* mutations in plasma, serum and tumor tissue samples of NSCLC patients. The objective of this study was to determine the feasibility and predictive utility of *EGFR* mutation detection in blood.

Patients and Methods

Patients

To be eligible for this study, patients were required to have pathologically confirmed NSCLC and available plasma, serum or tumor tissue for *EGFR* mutation analysis. 164 patients were enrolled in this study from October 2011 to October 2012 at Shanghai Pulmonary Hospital. Patients' clinicopathologic characteristics, treatment regimens, tumor responses and survival outcomes were recorded. Smoking history was based on records at patients' first clinic visit and having smoked more than 100 cigarettes in a lifetime was used to define smokers. Performance status was evaluated using the Eastern Cooperative Oncology Group criteria. Tumor response was assessed according to the Response Evaluation Criteria in Solid Tumours guidelines. Written informed consent was obtained from all participants, and provision of plasma, serum and tumor tissue for *EGFR* mutation analysis was optional. This study was approved by the Institutional Ethics Committee of Shanghai Pulmonary Hospital.

Sample Collection

Plasma was collected from 141 patients and serum from 108 patients. Plasma/serum was separated from 4 mL peripheral blood by centrifugation at 1,000 rpm for 10 min at 4°C within 4 hours after collection and stored at -80°C until DNA extraction. Tumor tissue obtained from 142 patients via biopsy was put into RNAlater solution (Ambion, Austin, Texas, USA) and stored at -80°C until DNA extraction. All tumor tissue samples went through pathologic evaluation to confirm the diagnosis of NSCLC.

DNA Extraction

DNA was extracted from 1 ml plasma/serum or 2-20 mg tumor tissue. The DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was used to extract DNA according to the manufacturer's instructions. The concentration and purity of DNA were determined by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, USA). DNA extracted from tumor tissue was standardized to 1 ng/μL, whereas cfDNA extracted from plasma/serum was used for *EGFR* mutation analysis immediately without standardization.

EGFR Mutation Analysis

The Human *EGFR* Gene Mutations Fluorescence Polymerase Chain Reaction Diagnostic Kit (Amoy Diagnostics, Xiamen, China), which is based on ARMS technology, was used to detect the 19Del, L858R and T790M mutation according to the manufacturer's instructions. Briefly, all reactions were performed in 25 μL volumes including 4.7 μL of template DNA, 0.3 μL of Taq polymerase and 20 μL of reaction buffer mix. Real-time PCR was carried out using MX3000P real-time PCR machine (Stratagene, La Jolla, CA, USA) under following conditions: (1) initial denaturation at 95°C for 5 min, (2) 15 cycles of 95°C 25 s, 64°C 20 s and 72°C 20 s, (3) 31 cycles of 93°C 25 s, 60°C 35 s and 72°C 20 s with fluorescence FAM and HEX reading at 60°C of each cycle in phase 3. Data analysis was performed with MxPro v4.10 (Stratagene, La Jolla, CA, USA). Cycle threshold (Ct) represents the threshold at which the signal was detected above background fluorescence. ΔCt values were calculated as the difference between the mutation Ct and control Ct. Positive results were defined as follows: (1) Ct is lower than 26, (2) Ct is higher than 26 and ΔCt is lower than the cut-off ΔCt value (11 for 19Del and L858R, 7 for T790M).

Statistical Analysis

SPSS statistical software, version 17.0 (SPSS, Inc., Chicago, IL, USA) was used to analyze the data. The comparison of *EGFR* mutation rate among different sample types and the correlation between *EGFR* mutation status and clinicopathologic characteristics as well as response to EGFR-TKIs were evaluated using Chi-square test or Fisher's exact test. Cohen's kappa statistic and McNemar's test were used to analyze the concordance of *EGFR* mutation status between matched samples. Progression-free survival (PFS) with EGFR-TKIs treatment according to *EGFR* mutation status was estimated by Kaplan-Meier method and compared using log-rank test. A two-sided P value less than 0.05 indicated statistical significance.

Results

Patient Characteristics

In total, 164 Chinese patients with NSCLC were enrolled in this study from October 2011 to October 2012 at Shanghai Pulmonary

Table 1. Patient Characteristics.

Characteristics	No. of patients (n = 164)	Percentage (%)
Age (years)		
Median	58	
Range	32-81	
Gender		
Female	68	41.5
Male	96	58.5
Smoking history		
Never smoker	84	51.2
Smoker	80	48.8
Histology		
Adenocarcinoma	128	78
Squamous cell carcinoma	18	11
Adenosquamous carcinoma	5	3
NSCLC NOS	13	8
Stage		
IIIb	14	8.5
IV	131	79.9
Postoperative relapse	19	11.6
Performance Status		
0-1	151	92.1
2	9	5.5
3-4	4	2.4

NSCLC, non-small cell lung cancer; NOS, not otherwise specified.

Hospital and their clinicopathologic characteristics are listed in Table 1. During this study, 96 patients didn't receive EGFR-TKIs, 19 received EGFR-TKIs as first-line therapy, 32 as second-line therapy and 17 as third-line or subsequent therapy. Of 68 patients who received EGFR-TKIs, 51 had their samples collected before EGFR-TKIs treatment and 17 after PD to EGFR-TKIs.

EGFR Mutation Status

A total of 141 plasma samples, 108 serum samples and 142 tumor tissue samples were available for *EGFR* mutation analysis (Table 2). *EGFR* mutations were detected in 66 (46.5%) tumor tissue samples, of which 38 samples harbored a 19Del, 27 a L858R and 8 a T790M (concurrent with 19Del in 6 and L858R in one). 36 (25.5%) plasma samples exhibited *EGFR* mutations, including 22 with 19Del, 14 with L858R and 6 with T790M (concurrent with 19Del in 4 and L858R in one). One plasma sample exhibited both 19Del and L858R. In serum

samples, *EGFR* mutation rate was 22.2%. 24 mutation-positive serum samples included 14 with 19Del, 9 with L858R and 3 with T790M (concurrent with 19Del in 2). *EGFR* mutation rate was significantly higher in tumor tissue than in plasma (46.5% versus 25.5%, P < 0.001) and serum (46.5% versus 22.2%, P < 0.001).

Correlation between EGFR Mutation Status and Clinico-pathologic Characteristics

The correlation between *EGFR* mutation status and patients' clinicopathologic characteristics was summarized in Table 3. In tumor tissue, *EGFR* mutation status was correlated with patients' gender, smoking history and histology. *EGFR* mutation rate was significantly higher in females than in males (60.0% versus 36.6%, P = 0.006), in never smokers than in smokers (55.4% versus 36.8%, P = 0.026) and in patients with adenocarcinoma than in those with other histology (53.7% versus 23.5%, P = 0.002). In blood samples, *EGFR* mutation status was only associated with histology. Patients with adenocarcinoma had significantly higher mutation rate than those with other histology in both plasma (30.0% versus 9.7%, P = 0.022) and serum (26.7% versus 4.5%, P = 0.024).

Comparison of EGFR Activating Mutation Status in Different Sample Types

→ Plasma versus Tumor Tissue

EGFR mutation status was analyzed in 121 patients who provided plasma and matched tumor tissue samples (Table 4). 89 patients had identical *EGFR* mutation status in both plasma and tumor tissue, including 27 with activating mutations and 62 with wild type. Discrepant mutation results were observed: 29 patients with mutant tumors had no mutation in matched plasma, whereas 3 patients with mutant cfDNA had no mutation in corresponding tumor tissue. The concordance rate of *EGFR* mutation status between plasma and tumor tissue was 73.6% (89/121). Compared with tumor tissue, the sensitivity and specificity for *EGFR* mutation detection in plasma by ARMS was 48.2% (27/56) and 95.4% (62/65), respectively. The false negative rate was high: 51.8% (29/56) of patients with *EGFR* mutant tumor were identified as wild type in plasma.

Table 2. *EGFR* Mutation Status.

cfDNA *EGFR* mutation status	Tumor *EGFR* mutation status							
	19Del only	L858R only	T790M only	19Del and T790M	L858R and T790M	Wild type	Unknown	Total
Plasma								
19Del only	13	0	0	2	0	1	1	17
L858R only	0	9	0	0	1	1	1	12
T790M only	0	1	0	0	0	0	0	1
19Del and L858R	0	0	0	0	0	1	0	1
19Del and T790M	1	0	0	1	0	0	2	4
L858R and T790M	0	1	0	0	0	0	0	1
Wild type	14	12	1	2	0	61	15	105
Unknown	4	4	0	1	0	12	2	23
Total	32	26	1	6	1	76	22	164
Serum								
19Del only	7	0	0	3	0	1	1	12
L858R only	0	6	0	0	1	1	1	9
T790M only	0	0	0	0	0	0	1	1
19Del and T790M	1	0	0	1	0	0	0	2
Wild type	13	14	0	2	0	41	14	84
Unknown	11	6	1	0	0	32	6	56
Total	32	26	1	6	1	76	22	164

EGFR, epidermal growth factor receptor; cfDNA, circulating free DNA; Unknown, no sample available.

Table 3. Correlation between Clinical Characteristics and *EGFR* Mutation Status.

Characteristic	Tumor tissue (n = 142)			Plasma (n = 141)			Serum (n = 108)		
	Mutation	Wild type	P values	Mutation	Wild type	P values	Mutation	Wild type	P values
Age			0.352			0.269			0.133
≥65 years	13	20		6	27		3	23	
<65 years	53	56		30	78		21	61	
Gender			0.006			0.790			0.877
Female	36	24		16	44		11	37	
Male	30	52		20	61		13	47	
Smoking history			0.026			0.136			0.108
Never	41	33		23	52		17	44	
Current/former	25	43		13	53		7	40	
Histology			0.002			0.022			0.024
Adenocarcinoma	58	50		33	77		23	63	
Non-adenocarcinoma	8	26		3	28		1	21	
Stage			0.445			0.831			0.061
IIIb-IV	60	66		33	95		24	73	
Postoperative replase	6	10		3	10		0	11	
Performance Status			0.724			0.729			1.000
0-1	63	71		34	96		22	76	
≥2	3	5		2	9		2	8	

EGFR, epidermal growth factor receptor.

→ **Serum versus Tumor Tissue**

For *EGFR* mutation analysis 92 patients provided serum and matched tumor tissue samples (Table 4). 61 patients exhibited identical *EGFR* mutation status in both serum and tumor tissue, including 19 with activating mutations and 42 with wild type. Discrepant results were observed: 29 patients with mutant tumors had no mutation in corresponding serum, whereas 2 patients with mutant cfDNA had no mutation in matched tumor tissue. The concordance rate of *EGFR* mutation status between serum and tumor tissue was 66.3% (61/92). Compared with tumor tissue, the sensitivity and specificity of *EGFR* mutation detection in serum by ARMS was 39.6% (19/48) and 95.5% (42/44), respectively. The false negative rate was high: 60.4% (29/48) of patients with *EGFR* mutant tumor were identified as wild type in serum.

→ **Plasma versus Serum**

94 patients provided plasma and paired serum samples. 82 patients exhibited identical *EGFR* mutation status in both plasma and serum, including 17 with activating mutations and 65 with wild type. Discordant results were observed: 9 patients had mutant cfDNA in plasma but not in serum, whereas 3 patients had mutant cfDNA in serum but not in plasma. The concordance rate of *EGFR* mutation status between plasma and serum was 87.2% (82/94). The kappa coefficient of 0.657 was statistically significant (P < 0.001), whereas the McNemar's test showed no significant difference (P = 0.146).

Comparison of EGFR T790M Mutation Status in Different Sample Types

T790M was detected in 14 (8.5%) patients. Among them, one patient exhibited T790M concurrent with 19Del in matched plasma, serum and tumor tissue, whereas 10 patients had discrepant results between blood and tumor tissue.

Correlation between EGFR Mutation Status and Response to EGFR-TKIs

In 68 patients who received EGFR-TKIs, the correlation between *EGFR* mutation status and response to EGFR-TKIs was analyzed (Table 5). For tumor tissue, objective response rate (ORR) of patients with or without *EGFR* activating mutations was 68.4% (26/38) and 10.5% (2/19), respectively (P < 0.001). For plasma samples, ORR of patients with or without *EGFR* activating mutations was 68.4% (13/19) and 38.9% (14/36), respectively (P = 0.037). For serum samples, ORR of *EGFR* activating mutation positive and negative patients was 75.0% (12/16) and 39.5% (15/38), respectively (P = 0.017). ORR of patients with *EGFR* mutant tumor was consistent to that of patients with *EGFR* mutant cfDNA in plasma (P = 1.000) and serum (P = 0.751), whereas ORR of patients with wild-type tumor was significantly lower than that of patients with wild-type cfDNA in plasma (P = 0.028) and serum (P = 0.024).

Of 17 patients who provided samples after PD to EGFR-TKIs, 9 (52.9%) exhibited T790M concurrent with an *EGFR* activating mutation. In addition, one patient with L858R in tumor tissue but

Table 4. Comparison of *EGFR* Activating Mutation Status in Different Sample Types.

Sample	Tumor tissue		Total	Concordance rate	Kappa coefficient	McNemar's test	Sensitivity	Specificity	False positive rate	False negative rate	PPV	NPV
	Mutation	Wild type										
Plasma				73.6%	0.450(P < 0.001)	P < 0.001	48.2%	95.4%	4.6%	51.8%	90.0%	68.1%
Mutation	27	3	30									
Wild type	29	62	91									
Total	56	65	121									
Serum				66.3%	0.342(P < 0.001)	P < 0.001	39.6%	95.5%	4.5%	60.4%	90.5%	59.2%
Mutation	19	2	21									
Wild type	29	42	71									
Total	48	44	92									

EGFR, epidermal growth factor receptor; PPV, positive predictive value; NPV, negative predictive value.

Table 5. Correlation between *EGFR* Activating Mutation Status and Response To EGFR-TKIs.

Sample	*EGFR* activating mutation status	CR + PR	SD + PD	Total
Tumor Tissue	Mutation	26	12	38
	Wild type	2	17	19
	Total	28	29	57
Plasma	Mutation	13	6	19
	Wild type	14	22	36
	Total	27	28	55
Serum	Mutation	12	4	16
	Wild type	15	23	38
	Total	27	27	54

EGFR, epidermal growth factor receptor; CR, Complete Response; PR, Partial Response; SD, Stable Disease; PD, Progressive Disease.

T790M in plasma before EGFR-TKIs treatment directly experienced PD after 1.4 months.

### Correlation between EGFR Mutation Status and Survival

The correlation between *EGFR* mutation status and median PFS time in patients treated with EGFR-TKIs was assessed. For tumor tissue, PFS for patients with or without *EGFR* activating mutations was 13.6 months (95% confidence interval [CI], 9.9 to 17.3) and 2.1 months (95% CI, 0.8 to 3.4), respectively. The difference was statistically significant ($P < 0.001$, Figure 1A). For plasma samples, patients with *EGFR* activating mutations had a PFS of 7.9 months (95% CI, 1.6 to 14.1) compared with 6.1 months (95% CI, 2.7 to 9.6) for patients with wild-type *EGFR* ($P = 0.953$, Figure 1B). For serum samples, patients harboring *EGFR* activating mutations had a longer PFS of 7.9 months (95% CI, 6.5 to 9.2) than 5.7 months

(95% CI, 2.1 to 9.2) for patients without mutations ($P = 0.889$, Figure 1C). Moreover, PFS of patients with *EGFR* mutant tumors was consistent to that of patients with *EGFR* mutant cfDNA in plasma ($P = 0.094$) and serum ($P = 0.176$), whereas PFS of patients with wild-type tumor was significantly shorter than that of patients with wild-type cfDNA in plasma ($P = 0.023$) and serum ($P = 0.023$).

Further, all 68 patients received EGFR-TKIs were stratified into 4 subgroups based on their mutational genotypes: (1) positive for *EGFR* activating mutations in both tumor tissue and blood (n = 20), (2) positive for *EGFR* activating mutations in tumor tissue but negative in blood (n = 18), (3) positive for *EGFR* activating mutations in blood but negative in tumor tissue (n = 4), and (4) negative for *EGFR* activating mutations in both tumor tissue and blood (n = 26). PFS for each group was graphed in Figure 1D. Patients in subgroup two had a favorable PFS of 19.7 months (95% CI, 11.5 to 28.0), compared with 11.0 months (95% CI, 3.1 to 19.0) of those in subgroup one ($P = 0.102$) and 1.7 (95% CI, 0.9 to 2.5) months of those in subgroup three ($P < 0.001$). Patients in subgroup four had a comparable PFS of 2.3 months (95% CI, 0.3 to 4.3) with those in subgroup three ($P = 0.508$).

Discussion

EGFR mutation analysis is recommended in clinical practice to direct personalized management for NSCLC patients. This study demonstrates the possibility of using blood to detect *EGFR* mutations, though tumor tissue remains the best sample.
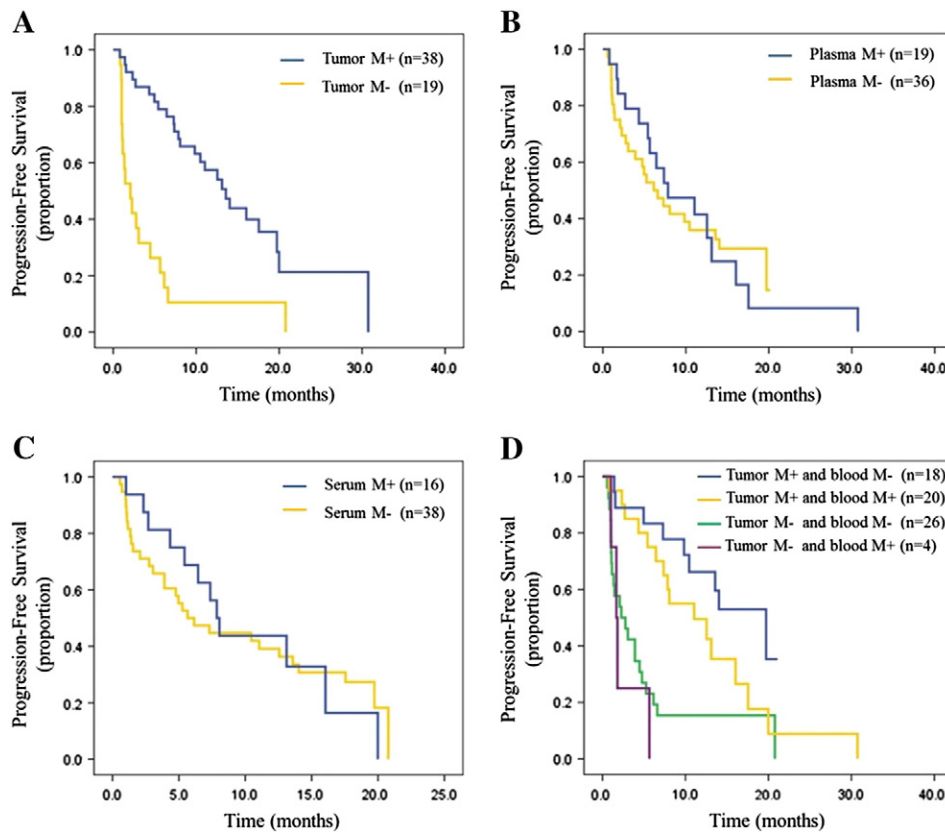
Figure 1. Progression-free survival (PFS) curves for 68 patients treated with epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors. A, PFS by *EGFR* activating mutation status in tumor tissue. B, PFS by *EGFR* activating mutation status in plasma. C, PFS by *EGFR* activating mutation status in serum. D, PFS by *EGFR* activating mutation status in both tumor tissue and blood samples. M+, positive for *EGFR* activating mutations; M-, negative for *EGFR* activating mutations.

The concordance of *EGFR* mutation status between blood and tumor tissue has been reported to be varying from 58.3% to 93.1%, with minimal false positive rate and variable false negative rate [17–21]. This study showed that compared with matched tumor tissue the concordance rate in plasma and serum was 73.6% and 66.3%, respectively. ARMS for *EGFR* mutation detection in cfDNA showed low sensitivity but high specificity. High specificity led to low false positive rate, suggesting that *EGFR* mutations identified in blood may be highly predictive of identical mutations in corresponding tumor. Low sensitivity caused high false negative rate, which was responsible for the significantly lower *EGFR* mutation rate in blood compared with tumor tissue. Thus, *EGFR* mutation-negative results in blood should be interpreted with caution as more than half of patients with *EGFR* mutant tumors were not detected in cfDNA by ARMS.

It is notable that 41 patients with mutant tumors had no detectable *EGFR* mutations in matched blood samples. This phenomenon has been observed in previous studies [18,22,23]. The trace amount and low percentage of mutant cfDNA could be below the detection limit of ARMS, giving rise to false negative results in blood. Another possible explanation is that prolonged storage of blood samples resulted in a decrease in the quantity of DNA extracted, thus affecting *EGFR* mutation detection [24]. In contrast, 5 patients with mutant cfDNA had no corresponding mutations in matched tumor tissue. This phenomenon has also been reported and could be explained by tumor heterogeneity: these biopsied tumor tissue samples may not carry the *EGFR* mutations detected in blood, because these mutations come from different parts of the tumor [25–27]. However, 4 of these 5 patients received EGFR-TKIs and had a comparable PFS with those who exhibited wild type in both blood and tumor tissue, suggesting that these mutations detected in blood could be false positive results.

There have been a limited number of studies on the correlation between *EGFR* mutation status in cfDNA and efficacy of EGFR-TKIs [28–32]. Though the researchers tend to agree that *EGFR* activating mutations in cfDNA may be predictive of better response to EGFR-TKIs, they are still uncertain whether *EGFR* mutation status in cfDNA can predict survival benefit from EGFR-TKIs. In a subgroup analysis of IPASS, ORR was 75.0% (18/24) and 27.1% (19/70) with gefitinib in patients with or without *EGFR* mutant cfDNA, respectively. PFS was significantly longer with gefitinib than carboplatin/paclitaxel in the cfDNA mutant subgroup (hazard ratio [HR], 0.29; 95% CI, 0.14-0.60; P < 0.001) but not in the cfDNA wild-type subgroup (HR, 0.88; 95% CI, 0.61-1.28; P = 0.50) [22]. Xu et al. reported that an significant correlation between *EGFR* mutations status in plasma and tumor response to gefitinib was observed using ARMS but not denaturing high-performance liquid chromatography (DHPLC), whereas no association between *EGFR* mutation status in plasma and PFS or overall survival (OS) was observed no matter using ARMS or DHPLC [33]. Bai et al. detected *EGFR* mutations in plasma using DHPLC and found that about 62.2% of patients with *EGFR* mutations responded to gefitinib, whereas 37.8% of patients with wild-type *EGFR* also responded. They noted that patients with *EGFR* mutant cfDNA had a significantly longer PFS than those with wild-type cfDNA (11.1 months versus 5.9 months, P = 0.044), though no difference in OS was seen [25].

In the current study, patients with *EGFR* activating mutations in tumor tissue had significantly greater ORR and longer PFS with EGFR-TKIs, which accords with the finding of previous clinical trials [4–8]. Patients harboring *EGFR* activating mutations in cfDNA also

had significantly higher ORR, which was consistent to that of patients with mutant tumors. In addition, patients with mutant cfDNA tended to have longer PFS than those with wild-type cfDNA, though the difference was not significant. These data suggest that *EGFR* activating mutations detected in blood may be predictive of improved tumor response and survival benefit from EGFR-TKIs. But patients with wild-type cfDNA had significantly higher ORR and longer PFS than those with wild-type tumors due to the presence of false negative results, suggesting that *EGFR* mutation-negative results detected in blood by ARMS is inferior to that in tumor tissue with respect to predicting clinical outcomes.

This study showed that in patients with *EGFR* mutant tumors those with wild-type cfDNA tended to have prolonged PFS compared with patients harboring corresponding mutant cfDNA. Similarly, a subgroup analysis of EURTAC indicated that in European patients with advanced *EGFR* mutation-positive NSCLC who received erlotinib as first-line therapy, the presence of mutant cfDNA in serum was associated with reduced PFS (HR, 0.48; 95% CI, 0.22-0.97; P = 0.04) and OS (HR, 0.46; 95% CI, 0.25-0.84; P = 0.02) [34]. For patients who provided pretreatment samples, the presence of *EGFR* mutations in blood may correlate with severe tumor burden, which contributes to higher proportion of tumor-derived cfDNA. Zhao et al. and Zhang et al. found that there were more detectable *EGFR* mutations in plasma from patients with advanced disease or patients with poorly differentiated tumors [21,35]. Park et al. reported that tumor burden was predictive of inferior survival in NSCLC patients with *EGFR* mutant tumor who received gefitinib [36]. For patients who provided posttreatment samples, therapy-related *EGFR* mutation status shift from mutation to wild type may correlate with better response, thus affecting survival benefit. Yung et al. found that plasma concentrations of *EGFR* mutations could decline to undetectable level after EGFR-TKIs treatment in responsive patients [23]. Besides, Bai et al. reported that patients whose *EGFR* mutation status in cfDNA changed from mutant state to wild type after chemotherapy had significantly better clinical response [37]. Dowson et al. demonstrated that cfDNA could provide the earliest measure of treatment response [38]. Hence, serial changes of *EGFR* mutation status in cfDNA during follow-up period could be informative in monitoring treatment response and predicting survival benefit. However, novel ultrasensitive methods would be preferable, so that smaller changes in cfDNA mutation status can be monitored in a better way.

The secondary T790M mutation has been reported to be present in about half of NSCLC patients with acquired resistance to EGFR-TKIs and is usually concurrent with activating mutations, which is consistent with this study [39]. Rosell et al. and Su et al. reported that patients with T790M-positive tumors before EGFR-TKIs treatment had a shorter PFS than those having T790M-negative tumors [40,41]. In this study one patient, with L858R in tumor tissue but T790M in plasma before EGFR-TKIs treatment, directly experienced PD after 1.4 months. Sakai et al. reported that when patients under 65 years who had partial response to EGFR-TKIs were grouped according to their T790M mutation status in plasma, patients with T790M had a significantly shorter PFS than patients without T790M [42]. These data suggest that T790M mutations in cfDNA may aid in monitoring resistance and predicting efficacy of EGFR-TKIs.

There were several limitations of this study. The sample size was relatively small and samples were not well matched. Besides, this study was not specifically designed to evaluate EGFR-TKIs treatment. Notwithstanding its limitations, this study demonstrates that *EGFR* mutations detected in blood of NSCLC patients by ARMS may be highly predictive of identical mutations in

corresponding tumor, as well as showing correlations with tumor response and survival benefit from EGFR-TKIs. However, due to the method's low sensitivity in blood samples, tumor tissue remains the best sample for *EGFR* mutation analysis. Further investigations involving appropriate methodologies to decrease false negatives in cfDNA-based *EGFR* mutation analysis are warranted.

Acknowledgments

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