

Epidermal Growth Factor Receptor Mutations in Plasma DNA Samples Predict Tumor Response in Chinese Patients With Stages IIIB to IV Non–Small-Cell Lung Cancer

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ABSTRACT

Purpose

Mutations in the epidermal growth factor receptor (EGFR) kinase domain can predict tumor response to tyrosine kinase inhibitors (TKIs) in non–small-cell lung cancer (NSCLC). However, obtaining tumor tissues for mutation analysis is challenging. We hypothesized that plasma-based EGFR mutation analysis is feasible and has value in predicting tumor response in patients with NSCLC.

Patients and Methods

Plasma DNA samples and matched tumors from 230 patients with stages IIIB to IV NSCLC were analyzed for EGFR mutations in exons 19 and 21 by using denaturing high-performance liquid chromatography. We compared the mutations in the plasma samples and the matched tumors and determined an association between EGFR mutation status and the patients' clinical outcomes prospectively.

Results

In 230 patients, we detected 81 EGFR mutations in 79 (34.3%) of the patients' plasma samples. We detected the same mutations in 63 (79.7%) of the matched tumors. Sixteen plasma (7.0%) and fourteen tumor (6.1%) samples showed unique mutations. The mutation frequencies were significantly higher in never-smokers and in patients with adenocarcinomas ($P = .012$ and $P = .009$, respectively). In the 102 patients who failed platinum-based treatment and who were treated with gefitinib, 22 (59.5%) of the 37 with EGFR mutations in the plasma samples, whereas only 15 (23.1%) of the 65 without EGFR mutations, achieved an objective response ($P = .002$). Patients with EGFR mutations had a significantly longer progression-free survival time than those without mutations ($P = .044$) in plasma.

Conclusion

EGFR mutations can be reliably detected in plasma DNA of patients with stages IIIB to IV NSCLC and can be used as a biomarker to predict tumor response to TKIs.

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INTRODUCTION

Lung cancer is the leading cause of cancer-related death in the world, particularly in major Chinese cities.¹ It is estimated that, by 2025, more than one million Chinese people will have died of lung cancer.² Non–small-cell lung cancer (NSCLC) is the most common histologic type, which affects approximately 80% of all patients with lung cancer.³ Because more than 70% of patients with lung cancer are diagnosed with advanced-stage disease, systemic treatment plays a central role in clinical management. However, routine platinum-based chemotherapy prolongs median survival times by only a few months in these patients compared with supportive

care. Currently, median survival times are about 9 and 18 months for patients with metastatic and locally advanced diseases, respectively.

Epidermal growth factor receptor (EGFR) is frequently overexpressed in NSCLC and is a promising target for therapy.⁴ Tyrosine kinase inhibitors (TKIs) that target EGFR, such as gefitinib, have demonstrated effectiveness in patients with refractory NSCLC,⁵⁻⁷ but only a fraction of patients respond to it. EGFR TKI sensitivity has been associated with never-smokers, women, Asian ethnicity, and adenocarcinoma histology; however, high EGFR protein levels are not associated with EGFR TKI sensitivity.⁸ In 2004, three groups independently reported a correlation between mutations in

the EGFR gene's kinase domain and sensitivity to EGFR TKIs.⁹⁻¹¹ Most EGFR mutations are observed in exons 19 and 21 and are either small in-frame deletions (delE746-751) or heterozygous mutations around the adenosine triphosphate binding pocket. The correlation between EGFR mutations and EGFR TKI sensitivity has been validated subsequently in several clinical trials.¹²⁻¹⁴

This major scientific breakthrough raises the question of whether EGFR mutations can be used as a biomarker to select patients for treatment with EGFR TKIs. Such selection is particularly important in China because of China's large patient population, limited national resources, and higher rate of EGFR mutation frequencies in the population.

EGFR mutations can be readily detected in primary tumors. However, it may be difficult to obtain tumor tissues for such analysis, particularly from patients with refractory NSCLC. Even in prospectively conducted clinical trials, less than 50% of the patients had tumors that were available for mutation analysis.¹⁵ Because plasma samples of patients with NSCLC often contain circulating DNA derived from tumor tissues, plasma samples have been used as surrogate tumor tissues for detecting genetic alterations.¹⁶⁻¹⁹ Several groups have demonstrated that EGFR mutations identical to those in the corresponding tumors can be detected in serum DNA samples.^{20,21} However, these studies were retrospective and had relatively small numbers of patients.

Therefore, in this single-center, prospective study, we analyzed 230 consecutive Chinese patients with stages IIIB to IV NSCLC for EGFR mutations in exons 19 and 21 by using both plasma samples and matched tumor tissues to determine the utility of plasma as a surrogate tissue for EGFR mutation analysis. We also analyzed correlations between EGFR mutation status and response to gefitinib treatment, as well as other clinical/pathologic parameters, to determine the potential clinical implications of EGFR mutation analysis in Chinese patients.

PATIENTS AND METHODS

Patient Population

To be eligible for the study, patients were required to have pathologically confirmed stage IIIB or IV NSCLC, an Eastern Cooperative Oncology Group performance status of 0 to 2, and available plasma and primary tumor tissue. Only patients treated at the Beijing Cancer Hospital from April 2004 to January 2007 were enrolled. The patients were prospectively observed for tumor responses and survival outcomes. Laboratory data were obtained and recorded independently by investigators blinded to clinical data until analyses were done by a biostatistician. The study was reviewed and approved by the Institutional Ethic Committee at Beijing Cancer Hospital. All the patients signed informed consent to participate in this study and gave permission for the use of their plasma and tumor tissues. Smoking status was based on records at patients' first clinic visit and having smoked greater than 100 cigarettes in a life time was used to define smokers.

The study was specifically designed to test a potential use of plasma instead of tumor tissues in EGFR mutation analysis and a potential predictive value in predicting clinical responses to EGFR treatment. The end point was tumor response rate to gefitinib. It was designed to detect a greater than 40% difference in response rate between samples with and without EGFR mutation with the assumption that 30% of Chinese patients with NSCLC carry EGFR mutations in the kinase domain. Twenty-six and 61 patients (N = 87) were required in mutant and wild-type arms, respectively, to provide a two-sided significance level of .05 and a power of .90 with which to detect a statistically significant difference. Because 35% to 40% of the Chinese patients would be

receiving gefitinib as second-line treatment in our institution, the sample size was calculated between 218 and 249. For tumor response assessment, we evaluated objective response after 8 weeks of treatment on the basis of computed tomography (CT) scans. We had a single radiologist (L.W.) assess all films while blind to EGFR mutation status.

Specimen Collection and DNA Extraction

We collected plasma before patients received second-line chemotherapy or gefitinib treatment (except for 24 patients who were on first-line treatment) and assessed corresponding tumors for DNA extraction. The tumors were macrodissected, and tumor contents were recorded for each sample by using immediately adjacent sections. The tumor content was greater than 30% in 78% of the tumor samples but was 20% to 30% in other samples.

Denaturing High-Performance Liquid Chromatography

We performed denaturing high-performance liquid chromatography (DHPLC) by using the Transgenomic Wave Nucleic Acid Fragment Analysis System with a DNasep column (Transgenomic, Omaha, NE). The mobile phases comprised 0.05% acetonitrile in 0.1 M triethylammonium acetate (TEAA; eluent A) and 25% acetonitrile in 0.1 M TEAA (eluent B). The polymerase chain reaction (PCR) products of exons 18, 20, and 21 were denatured at 95°C for 5 minutes and were cooled to 35°C at a rate of 1°C per minute to allow formation of heterozygote DNA. The product of exon19 did not need to be denatured. The flow rate was 0.9 mL/min, and an ultraviolet detector was set at 260 nm. We identified the heterozygous profiles by visual inspection of the chromatograms on the basis of the appearance of additional, earlier-eluting peaks. Corresponding homozygous profiles showed only one peak.

To determine the detection limit of DHPLC, we used four plasmids that contained the deletion mutation (delE746-A751) in exon19, L858R mutation in exon21, and wild-type exon19 and 21 sequences. Serial dilutions (50%, 25%, 12.5%, 6.25%, 3.125%, and 1.6% of mutant alleles) were made for the DHPLC analysis.

Statistical Analysis

We used SAS statistical software, version 10.0 (SAS Institute, Cary, NC) to analyze the data, and we used the χ^2 and Fisher's exact tests to assess the relationship between EGFR gene mutation status and each of the clinical and pathologic parameters. A *P* value less than .05 was considered statistically significant. The 95% CIs for odds ratios and frequencies were calculated as exact CIs. The time to event variables (ie, duration of overall survival [OS] and progression-free survival [PFS]) and the median OS and PFS were calculated by Kaplan-Meier estimation.

RESULTS

Patient Characteristics

A total of 230 patients met the enrollment criteria and were entered onto the study from April 2004 to January 2007 at the Beijing Cancer Hospital. The patients consisted of 107 women and 123 men. There were 171 patients with lung adenocarcinomas, 55 with squamous cell carcinomas, and four with large-cell carcinomas. A total of 103 patients were smokers, and 127 were never-smokers. Twenty-one patients had previously undergone surgery for early-stage tumors, and the remaining 209 patients were initially diagnosed with stages IIIB to IV NSCLC. Two hundred six (89.6%) patients had received prior platinum-based chemotherapy, whereas 24 (10.4%) were treatment-naïve at the time of sample collection. During this study, all the patients received chemotherapy, and 102 patients received second-line gefitinib treatment. The patients' clinical and disease characteristics are listed in Table 1.

Pilot EGFR Mutation Analysis

Before we analyzed the samples of the study population, we performed a pilot experiment to standardize our methodology. We

Table 1. Patient Clinical and Disease Characteristics

Variable	No. of Patients (N = 230)	% of Patients
Age, years		
Mean	60.7	
Standard deviation	4.5	
Sex		
Male	123	53.5
Female	107	46.5
Smoking history		
Smoker	103	44.8
Never smoker	127	55.2
Histologic type		
ADC	171	74.3
SCC	55	23.9
LCC	4	1.8
Disease stage		
IIIB	80	34.8
IV	150	65.2
Therapy		
Chemotherapy	230	100
Gefitinib	102	44.3

Abbreviations: ADC, adenocarcinoma; LCC, large-cell carcinoma; SCC, squamous cell carcinoma.

used genomic DNA samples extracted from 60 frozen primary tumors that were surgically resected from patients with NSCLC to screen for mutations and small deletions in EGFR exons 18 to 21 by using DHPLC and to determine patterns of the mutations/deletions to serve as references. We found 12 deletion mutations in exon 19, seven L858R mutations in exon 21, one mutation in exon 20, but no mutation in exon 18. We confirmed these results by direct sequencing analysis. Because mutations/deletions were rarely observed in exon 18, we decided to emphasize exons 19 to 21 in our subsequent investigation.

On DHPLC analysis, the EGFR exon 19 deletion (delE746-751) exhibited two peaks. The eluting peak of the wild-type sequence arrived at 4 minutes 1 second, whereas the peak of the deletion mutant arrived at 3 minutes 11 seconds (Fig 1A). The EGFR exon 21 mutation

exhibited an abnormal overshoot at the peak of the wild-type (heterozygous peaks), whereas the wild-type alleles showed a sharp peak pattern (Fig 1B). The detection sensitivity was determined by using serial dilution experiment. The results showed that the detection limit of the DHPLC method is approximately 3% of the mutant alleles among normal gene copy background for both exon 19 and exon 21 mutations (Fig 1C and 1D).

EGFR Mutations in Circulating Plasma DNA and Matched Primary Tumor DNA

We used the DHPLC method established in our pilot experiment to analyze the 230 patients enrolled on this study. We detected 81 EGFR exon 19 or 21 mutations in the plasma samples of 79 (34.3%) patients, including 56 exon 19 deletion mutations and 25 exon 21 point mutations. (Two plasma DNA samples exhibited mutations at both exons 19 and 21.) No mutation was found in exon 20 in both plasma DNA and tumor tissue of the 230 patient cases. However, we noticed a common single nucleotide substitution at nucleotide 2361 (G/A), codon 787 (Q787Q), in 74 (32.2%) of the 230 patient cases.

In the 230 matched primary tumors, 79 mutations were detected in 77 (33.5%) of the tumors, including 53 in exon 19 and 26 in exon 21. Among the 79 patients with plasma DNA mutations, 63 (79.7%) had identical mutations detected in the matched tumor DNA, which indicated a high correlation between the mutations detected in plasma DNA and the mutations detected in the corresponding tumor DNA ($P < .001$; correlation index, 0.74). Interestingly, 16 (7.0%) patients with plasma DNA mutations had no detectable EGFR mutations in the corresponding primary tumors. Similarly, 14 patients with tumor DNA mutations had no detectable EGFR mutation in the corresponding plasma DNA samples. The correlation between mutations detected in plasma DNA and tumor DNA is listed in Table 2.

To verify the inconsistent EGFR mutations between the plasma samples and the primary tumor tissues, we analyzed six paired specimens (three with EGFR mutations in the plasma samples only and three with EGFR mutations in the primary tumor tissues only) by sequencing the DNA fragments in the individual clones from each DNA sample. The results were consistent with the DHPLC findings.

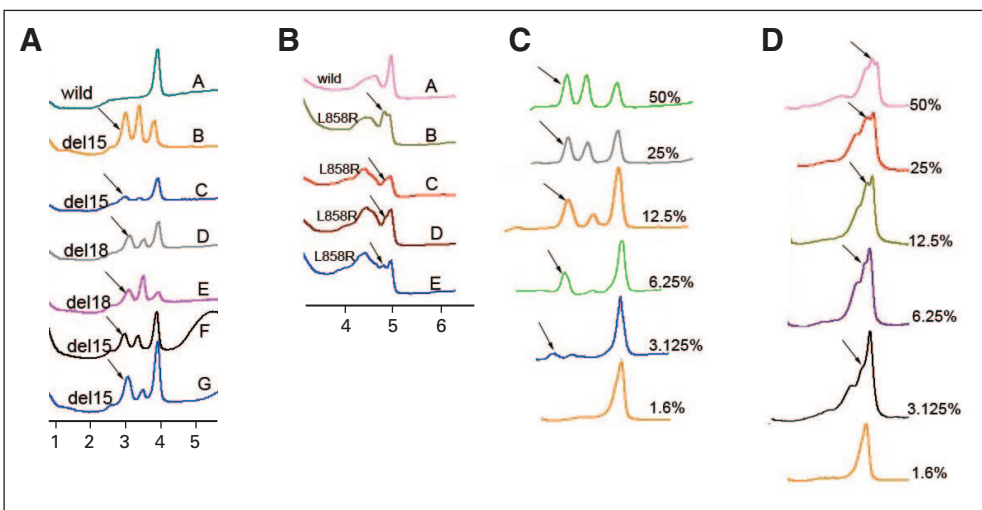


Fig 1. Epidermal growth factor receptor mutations in exons 19 and 21 detected by denaturing high performance liquid chromatography (DHPLC). (A) DHPLC patterns of exon 19 deletion mutations in non-small-cell lung cancer: (a) wild-type; (b-g) 15- or 18-base pair deletion mutations. (B) DHPLC pattern of exon 21 mutation in NSCLC: (a) wild-type; (b-e) L858R mutation. (C) Dilution analysis of exon 19 deletion mutation in a plasmid. The ratios of mutant to wild-type DNA are indicated. (D) Dilution analysis of exon 21 mutation. The ratios of mutant to wild-type DNA are indicated. The detect limit is approximately 3% in both exon 19 and exon 21. → indicate epidermal growth factor receptor mutation.

Table 2. Correlation of EGFR Mutations Between Plasma DNA and Primary Tumor DNA

Correlate	Tumor		Case Number
	EGFR-Positive	EGFR-Negative	
Plasma			
EGFR-positive	63	16	79
EGFR-negative	14	137	151
Case number	77	153	230*

Abbreviation: EGFR, epidermal growth factor receptor.

*Correlation index = 0.74.

Correlation Between EGFR Mutations and Clinicopathologic Characteristics

We analyzed whether there was a potential relationship between EGFR mutation status and the patients' clinicopathologic parameters. We found that EGFR mutation status in plasma DNA or in primary tumor DNA did not correlate with patients' age or sex or with disease stage. However, EGFR mutation status in both plasma DNA samples and primary tumor DNA did correlate with the patients' smoking history and tumor histology. There were significantly higher mutation rates in the never-smokers than in the smokers ($P = .012$ and $P = .017$ for plasma DNA and tumor DNA, respectively; Table 3). Similarly, patients with lung adenocarcinomas had higher mutation rates than those with other histologies ($P = .009$ and $P = .005$ for plasma DNA and tumor DNA, respectively; Table 3). Table 3 also lists the subgroup analyses of exon 19 and 21 mutations in plasma DNA, tumor DNA, and both plasma and tumor DNA.

Correlation Between EGFR Mutations and Gefitinib Response

Among the 230 patients, 102 had measurable tumors and received gefitinib as second-line treatment for at least two cycles. By using the Response Evaluation Criteria in Solid Tumors, 37 (36.3%) patients achieved a partial response (PR), which had a median duration of 10.6 months. The patients with EGFR mutations in either the plasma DNA or the tumor DNA achieved higher PR rates than those with wild-type EGFR. Among the 37 patients who achieved a PR, 23 (62.2%) and 22 (59.5%) had detectable EGFR mutations in either the tumor DNA or the plasma DNA, respectively. In contrast, among the 65 patients who did not achieve a PR, only 14 (21.5%) and 15 (23.1%) had detectable EGFR mutations in either the tumor DNA or the plasma DNA, respectively (tumor DNA: $P = .001$; 95% CI, 1.82 to 10.10; plasma DNA: $P = .002$; 95% CI, 2.04 to 11.76). In a multivariate analysis that considered tumor histology, smoking status, sex, and tumor stage as cofactors, EGFR mutation was the only independent factor of tumor response (Table 4).

Correlation Between EGFR Mutations and Survival

We also analyzed the potential implication of EGFR mutation status in predicting clinical outcome in the patients with NSCLC who received gefitinib. The follow-up time was calculated from the start of gefitinib treatment. The median follow-up time of these 102 patients was 25.7 months (range, 9.1 to 32.5 months); the median PFS time was 8.6 months; and the median OS time was 15.9 months. The median PFS time for patients with EGFR mutations was significantly longer than that for patients with wild-type EGFR. Patients with EGFR mutations in plasma DNA had a PFS time of 11.1 months (95% CI, 8.7 to

Table 3. Association Between EGFR Mutations and Clinicopathological Parameters

Item	Patients (N = 230)		T-E19 (n = 53)	T-E21 (n = 26)	Any T (n = 77)	P-E19 (n = 56)	P-E21 (n = 25)	Any P (n = 79)	Any PT (n = 95)
	No.	%							
Age, years									
≤ 60	111	48.3	29	14	41	31	13	43	45
> 60	119	51.7	24	12	36	25	12	36	50
P		—	.284	.545	.283	.222	.692	.176	.820
Sex									
Female	107	46.5	24	13	37	26	14	39	44
Male	123	53.5	29	13	40	30	11	40	51
P		—	.837	.706	.741	.987	.314	.531	.958
Smoking									
Smokers	103	44.8	17	8	25	19	8	26	33
Never smokers	127	55.2	36	18	52	37	17	53	62
P		—	.061	.127	.017	.060	.173	.012	.010
Pathology									
ADC	171	74.3	45	22	66	47	20	67	83
Non-ADC	59	25.7	8	4	11	9	5	12	12
P		—	.045	.203	.005	.051	.493	.009	< .0001
Disease stage									
IV	150	65.2	32	19	49	31	17	46	59
IIIB	80	34.8	21	7	28	25	8	33	36
P		—	.399	.372	.721	.075	.757	.107	.406

Abbreviations: EGFR, epidermal growth factor receptor; T-E19, exon 19 mutation status in tissue DNA; T-E21, exon 21 mutation status in tissue DNA; Any T, total EGFR mutations in tissue DNA; P-E19, exon 19 mutation status in plasma DNA; P-E21, exon 21 mutation status in plasma DNA; Any P, total EGFR mutations in plasma DNA; Any PT, total mutations positive in tissue or plasma DNA; ADC, adenocarcinoma.

Table 4. Association Between Clinical Features and Tumor Responses Using Multivariate Analysis

Parameter	Multivariate Analysis		
	P	OR	95% CI
Mutation	.001	5.525	2.101 to 14.493
Histology	.981	0.981	0.265 to 3.903
Sex	.507	1.972	0.484 to 4.329
Age	.765	1.148	0.465 to 2.832
Tumor stage	.057	2.911	0.909 to 9.065
Smoking status	.714	1.253	0.374 to 4.199

Abbreviation: OR, odds ratio.

16.8) compared with 5.9 months (95% CI, 2.1 to 9.7) for the patients with no EGFR mutations ($P = .044$ by log-rank test; Fig 2A). Similar results were obtained when we stratified the data by EGFR mutation status in tumor DNA ($P = .023$ by log-rank test; Fig 2B). An association between EGFR mutation status and OS was not observed in either plasma DNA- or tumor DNA-based analyses.

DISCUSSION

Our study demonstrated the possibility of using plasma/serum DNA as a surrogate tissue to measure EGFR mutation status. In addition, we found that EGFR mutation status in the surrogate tissue could predict

tumor response to gefitinib treatment, which is not surprising, as most mutations (approximately 80%) were detected in both the plasma DNA and the corresponding tumor DNA samples.

However, it is notable that we found EGFR mutations in either the plasma DNA samples only or the tumor DNA samples only in 13.0% of our patients (7% in each tissue type). This phenomenon has also been observed in previous studies that had smaller sample sizes.^{20,21} One possibility for this inconsistency in mutation status is the heterogeneity of genetic abnormalities in the tumors. In such instances, tumor biopsy specimens might not carry the EGFR mutations detected in circulating plasma DNA, because these mutations could come from different parts of the tumor. The lower tumor cell content in some of the tumors might also contribute to the lack of detectable mutations. However, all the tumors contained at least 20% tumor cells. Considering the high detection sensitivity of the method used (approximately 3% mutant alleles), this is an unlikely explanation. Likewise, if the tumor parts that carried mutations shed less DNA than the other parts of the tumors into plasma, evaluation may miss such mutations in plasma DNA. The dilution of DNA derived from non-cancerous tissues, such as inflamed tissues, might impede the detection of mutations in plasma DNA, despite the presence of mutations in tumors. Nevertheless, the inability to obtain primary tumor tissues, particularly through repeat biopsy, from patients with advanced-stage lung cancer makes the use of plasma/serum as a surrogate tissue for genetic analysis clinically important.

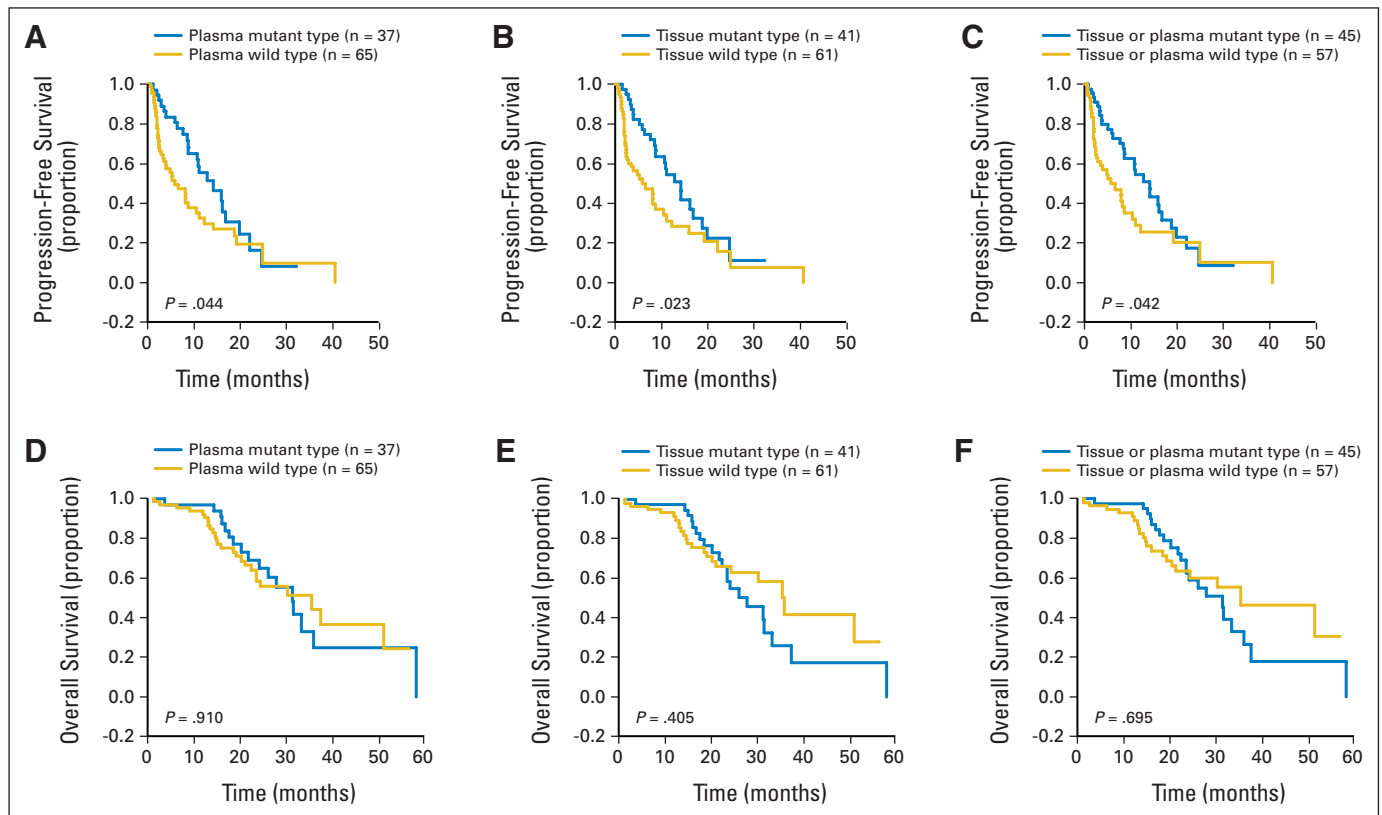


Fig 2. Progression-free survival (PFS) curves for the 102 patients treated with gefitinib. (A) PFS by epidermal growth factor receptor (EGFR) mutation status measured in circulating plasma DNA. (B) PFS by EGFR mutation status measured in tumor tissues. (C) PFS by EGFR mutation status measured in either tumor tissues or plasma DNA. (D) Overall survival (OS) by EGFR mutation status measured in plasma DNA. (E) OS by EGFR mutation status measured in tumor tissues. (F) OS by EGFR mutation status measured in either tumor tissues or plasma DNA.

A number of factors will impact the use of genetic tests in routine clinical practice, including the technical complexity, turnaround time, and cost of the tests. These factors are particularly important for countries such as China, where the patient population is large and health care resources are limited. The DHPLC method utilized in this study is technically easier and less expensive and has a quicker turnaround time than sequencing analysis. It has been used for EGFR mutation analysis by other investigators and showed better detection sensitivity than DNA sequencing.^{22,23} Our results were consistent with those from that study^{22,23} and support the utility of this technology in routine clinical practice.

Like others,⁵⁻⁷ we showed that EGFR mutations are more common in females and never-smokers. The difference in our study was that our population consisted solely of Chinese patients, whereas most of the other studies had a limited number of Chinese patients. Because of the relatively large sample size in our study, we concluded that the EGFR mutation rate of 35% in our study was representative of advanced-stage NSCLC in major Chinese cancer centers. Also, the characteristics of our study population were similar to the entire cohort treated at Beijing Cancer Hospital during the past 5 years (data not shown).

Our findings of a correlation between EGFR mutations and tumor response to TKI treatment and such treatment's lack of impact on OS were also consistent with previously reported data.^{24,25} In our patient population, about 62.2% of the tumors with EGFR mutations responded to gefitinib, whereas 37.8% of the tumors without the mutations also responded. Although no difference in OS was seen between patients with or without EGFR mutations, patients with EGFR mutations had significantly longer PFS times after gefitinib treatment, which suggests that these patients might have benefited from the treatment. It should be noted that our study was not specifically designed to test gefitinib treatment and that many patients received other chemotherapeutic agents, which makes data interpretation difficult. Additional clinical studies with specifically defined treatment regimens and larger sample sizes are necessary.

In summary, our study provides strong evidence to suggest that circulating plasma DNA may be used as a surrogate tissue for EGFR

mutation analysis in NSCLC. We are currently planning a prospective, multicenter study to validate the findings and to determine the value of the assay in predicting patients' responses to TKI. Once validated in additional clinical trials, our strategy could allow rapid analysis of circulating plasma/serum DNA for molecular assessment to generate information necessary for patient selection in personalized cancer management.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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