Comparison of Epidermal Growth Factor Receptor Mutation Statuses in Tissue and Plasma in Stage I–IV Non-Small Cell Lung Cancer Patients

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Key Words

Epidermal growth factor receptor • Mutation • Non-small cell lung cancer • Plasma

Abstract

Background: Epidermal growth factor receptor (EGFR) mutations play essential roles in the treatment of non-small cell lung cancer (NSCLC) patients using EGFR tyrosine kinase inhibitors. Detection of EGFR mutations in blood cell-free DNA (cfDNA) seems promising. However, the mutation status in the plasma/serum is not always consistent with that in the tissues. **Objectives:** The aims of this study were to compare the mutation statuses in plasma to those in tissues and thus to determine the specific subgroups of NSCLC patients who may be the best candidates for EGFR mutation analyses using blood cfDNA. **Methods:** A total of 111 pairs of tissue and plasma samples were collected. Mutant-enriched PCR and sequencing analyses were performed to detect EGFR exon 19 deletions and exon 21 L858R mutations. **Results:** Mutations were discovered in 43.2% (48/111) of the patients. The overall rate of consistency of the EGFR mutation statuses for the 111 paired plasma and tissue samples was 71.2% (79/111). The sensitivity and specificity rates of detecting EGFR mutations in the plasma were 35.6% (16/45) and 95.5% (63/66),

respectively. The disease stage and tumor differentiation subgroups showed significantly different detection sensitivities; the sensitivity was 10% in early-stage patients and 56% in advanced-stage patients ($p = 0.0014$). For patients with poorly differentiated tumors, the sensitivity was 77.8%, which was significantly different from those with highly differentiated (20%; $p = 0.0230$) and moderately differentiated tumors (19%; $p = 0.0042$). **Conclusion:** Blood analyses for EGFR mutations may be effectively used in advanced-stage patients or patients with poorly differentiated tumors.

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Introduction

Epidermal growth factor receptor (EGFR) activating mutations play essential roles in the treatment of non-small cell lung cancer (NSCLC) patients using EGFR tyrosine kinase inhibitors (TKIs) [1–3]. The detection of EGFR mutations in tissue has been considered to be the gold standard in the prediction of TKI treatment responses and prognoses. Unfortunately, tissue specimens are sometimes limited [4]. Elevated plasma or serum cell-free DNA (cfDNA) levels in lung cancer patients have been previously reported in the literature [5–7]. However,

cfDNA-associated mechanisms have not yet been clarified. It is possible that tumor DNA is released into the blood circulation via cell apoptosis or necrosis [8]. However, the detection of EGFR mutations in cfDNA has been reported to be feasible in NSCLC patients. Kimura et al. [9] first reported the utility of cfDNA for EGFR mutation screening and the high associated consistencies of treatment responses to TKIs. Disappointingly, EGFR mutation statuses in the plasma and/or serum were not always consistent with those in tissues. Although the use of plasma to detect EGFR mutations has been validated, few studies have reported on the association between the consistency of detection and the grouping of patients according to their clinical characteristics. The identification of subgroups that have higher consistent EGFR mutation statuses in both plasma and tissues would enable the selection of enriched populations for blood EGFR mutation testing.

Approximately 30 activating mutations in exons 18–21 of the EGFR gene have been reported [1, 2, 10, 11]. The in-frame deletions of exon 19 and the point mutation replacing leucine with arginine at codon 858 in exon 21 are commonly observed in NSCLC; these mutations account for 90% of the mutations, which could explain the dramatic responses to gefitinib and/or erlotinib [11–14]. In this study, EGFR exon 19 deletions and exon 21 L858R mutations in paired tissue and plasma samples were analyzed by mutant-enriched PCR (ME-PCR).

The aims of this study were to compare the mutation statuses in the plasma with those in tissues and thus to determine the specific subgroups of patients who may be the best candidates for EGFR mutation analyses using blood cfDNA.

Material and Methods

Patients and Materials

NSCLC patients who were newly diagnosed from December 2008 to September 2010 at the Department of Respiratory Diseases, Peking Union Medical College Hospital, and the Department of Thoracic Surgery, People's Hospital, Peking University, with available tissue and blood specimens were enrolled in this study. All samples were collected from the remains of clinical diagnoses. The study was approved by the Peking Union Medical College Hospital Institutional Review Board. Written informed consent was obtained from all the patients.

A total of 111 pairs of formalin-fixed, paraffin-embedded tissue and blood specimens were collected from Peking Union Medical College Hospital and the People's Hospital, Peking University, before the patients were treated. Tissue specimens were obtained by transbronchial/percutaneous biopsies (58) and from various operations (53). All of the tissue specimens underwent histologi-

cal examination by pathologists from Peking Union Medical College Hospital to confirm the NSCLC diagnoses, and the associated plasma specimens were also collected. For each patient, 200 µl of plasma were collected and stored at –80°C until use. The interval between tissue collection and that of the paired blood specimen was less than 1 week.

DNA Extraction

Formalin-fixed, paraffin-embedded slices were deparaffinized in xylene and rehydrated in descending grades of ethanol. DNA was extracted using the Magnetic Genomic DNA Kit (Tiangen Biotech, Beijing, China), according to the manufacturer's protocol. DNA concentrations were quantified using the NanoDrop ND-1000 (NanoDrop Technologies, Rockland, Del., USA). The extracted DNA was stored at –20°C until use.

ME-PCR Analyses for EGFR Mutations in Exons 19 and 21

ME-PCR is a highly sensitive assay for the detection of EGFR mutations [15]. In this study, we followed the methodology of Asano et al. [15] with some modifications.

Detection of Exon 19 Deletions

The sequences of primers for the first-round PCR amplification were as follows: 5'-ATCCCAGAAGGTGAGAAAGATAAA-ATTC-3' (forward primer, 19F1) and 5'-ACATTTAGGATGTG-GAGATGAGCAG-3' (reverse primer, 19R1). In this assay, the first round of amplification was conducted for 20 cycles (30 s at 95°C, 30 s at 60°C and 30 s at 72°C for 5 cycles, then 30 s at 95°C, 30 s at 55°C and 30 s at 72°C for 15 cycles) using 5–100 ng of sample DNA, 5 pmol of each primer and 3 µl of GoTaq Colorless Master Mix (Promega, Valencia, Calif., USA). We used *MseI* (NEB, Beijing, China) to digest the wild-type sequence TTAA in exon 19. Because the TTAA sequence is located 26 bases upstream of the first letter of codon 747, which also occurs in the mutant genes, a forward mismatch primer was designed to create a 1-base-mismatched sequence (ATAA, T to A) at this site to prevent *MseI* digestion at the primer-attaching site in the amplicon. Intermittent restriction digestions of 1 µl of the first PCR product were performed using 2.5 IU of *MseI* at 37°C for 4 h. After digestion, a 2-µl aliquot was withdrawn from the 20-µl solution to be used as a template for the second round of PCR amplification for 40 cycles (30 s at 95°C, 30 s at 60°C and 30 s at 72°C). The nested forward and reverse primers for the second round were as follows: 5'-AGGTGAGAAAGATAAAATTCCTGTC-3' (forward primer, 19F2) and 5'-GAGATGAGCAGGGTCTAGAGCAG-3' (reverse primer, 19R2).

Detection of the L858R Mutation

The analysis of L858R was essentially the same as that of exon 19 with the exception of the primers and restriction enzyme. Two pairs of nested primers were used. The sequences were as follows: 5'-TCAGAGCCTGGCATGAACATGACCCTG-3' (forward primer, 21F1) and 5'-GGTCCCTGGTGTCAGGAAAATGCTGG-3' (reverse primer, 21R1), and 5'-CAGCAGGGTCTTCTCTGTTT-TC-3' (forward primer, 21F2) and 5'-GAAAATGCTGGTGACCTAAAG-3' (reverse primer, 21R2). *MseI* was used to digest the wild-type gene. The PCR amplification and restriction digestion protocols for exon 21 were the same as those for exon 19.

Each sample was amplified in three independent PCR reactions. The products of the second amplification were purified and

Table 1. Characteristics of the patients with NSCLC before treatment

	n	EGFR mutation status in tissue	
		+	–
Gender			
Female	35 (31.5)	20	15
Male	76 (68.5)	25	51
Age			
≥60 years	59 (53.2)	24	35
<60 years	52 (46.8)	21	31
Smoking history			
Ever	57 (51.4)	17	40
Never	54 (48.6)	28	26
Disease stage			
I	22 (19.8)	11	11
II	10 (9.0)	1	9
IIIA	19 (17.1)	8	11
IIIB	14 (12.6)	4	10
IV	46 (41.4)	21	25
Histopathology type			
Squamous cell	35 (31.5)	2	33
Adenocarcinoma	73 (65.8)	43	30
Other types of NSCLC	3 (2.7)	0	3
Differentiation			
High	20 (18.0)	10	10
Moderate	45 (40.5)	21	24
Poor	34 (30.6)	9	25
Undetermined	12 (10.8)	5	7
Total	111 (100)	45	66

Figures in parentheses represent percentages.

sequenced (in the reverse orientation for exon 19 and in both the forward and reverse orientations for exon 21) by Invitrogen (Shanghai, China) or Sangon Biotech (Shanghai, China) Co., Ltd. The sequences were compared with the GenBank-archived human EGFR sequence (accession No. AY588246) and read by two investigators independently. In cases of discordance, a third observer also performed an analysis and made the final decision. Laboratory data were obtained and recorded independently by investigators who were blinded to the clinical data until analyses were conducted by a statistician.

Statistical Analyses

The SAS statistical software, version 9.0 (SAS Institute, Cary, N.C., USA) was used to analyze the data. McNemar's test was used to assess the significance of the difference between the mutation detection rates in the tissue and plasma samples. The degree of agreement was measured by the kappa test. EGFR mutations in tissue samples were considered to be the gold standard for the sensitivity and specificity measurements. All of the categorical variables were analyzed with the χ^2 test or Fisher's exact test. A *p* value of less than 0.05 was considered to be statistically significant.

Results

Patient Clinical Characteristics

The clinical characteristics of the patients are summarized in table 1. Of the 111 patients who were enrolled into this study, 35 were women and 76 were men, with a median age of 59 years (range 27–87 years). There were 57 ever smokers and 54 never smokers. Smoking status was based on records from the patients' first clinic visits, and an ever smoker was defined as a person who had smoked greater than 100 cigarettes in his/her lifetime. Disease stage was determined according to the 7th edition of the *TNM Classification of Malignant Tumors* [16]. Twenty-two patients were classified as stage I, 10 as stage II, 33 as stage III and 46 as stage IV. Histological types were determined according to the 3rd World Health Organization/International Association for the Study of Lung Cancer classifications, with 73 being classified as adenocarcinomas, 35 as squamous cell carcinomas and 3 as other types of NSCLC. Among the 111 tissue samples, 20 were highly differentiated, 45 were moderately differentiated, 34 were poorly differentiated and 12 were unable to be assessed.

Sensitivity Assays

We constructed a genomic DNA series with mutant DNA gradients using the DNA extracted from the three cell lines A549 (wild-type), H1975 (L858R) and H1650 (del746–750). The concentration that was used for the series was 60 ng/μl. This assay revealed that ME-PCR was able to detect a mutant molecule that was present in a background of wild-type sequences at a proportion of 0.2%. The mutant DNA could not be detected at a proportion of less than 0.2%, but it could still be detected even when the template was diluted to 5 ng/μl at 0.2%.

EGFR Mutation Rates in Tissues and Plasma

Table 2 shows the EGFR mutations that were detected in the tissues and plasma. Forty-five tissue samples (40.5%) and 19 plasma samples (17.1%) presented EGFR mutations. Among the patients with EGFR mutations in their tissue samples, 52% had exon 19 deletions and 48% had L858R mutations. Of the patients with the plasma EGFR mutations, 55% had exon 19 deletions and 45% had L865R mutations. One patient harbored exon 19 and exon 21 mutations in both the tissue and plasma samples.

The Correlation of EGFR Mutation Status in Tissue and Plasma Samples

Of the 111 paired samples, 79 presented the same EGFR mutation statuses in the tissues and plasma. Three

Table 2. Summary of EGFR mutations in tissue and plasma samples

	M-E19	M-E21	M-E19 or M-E21
Tissue	24 (21.6)	22 (19.8)	45 (40.5)
Plasma	11 (9.9)	9 (8.1)	19 (17.1)
Tissue or plasma	26 (23.4)	23 (20.7)	48 (43.2)

Figures represent numbers (percentages). M-E19 = Mutation in exon 19; M-E21 = mutation in exon 21.

plasma samples tested positive for EGFR mutations but showed negative results in the paired tissue samples, whereas 29 were positive in the tissues but negative in the plasma samples. The mutation rates in the tissues and plasma were significantly different as assessed by McNemar's test ($\chi^2 = 21.125$, $p < 0.001$). The degree of agreement for the testing of EGFR mutations in tissues and plasma was revealed to be weak by the kappa test, which showed a coefficient of 0.342 [95% confidence interval (CI) 0.182–0.501]. The correlations between the mutations that were detected in the tissues and plasma are listed in table 3.

Consistencies, Sensitivities and Specificities Achieved Using Plasma and Associated Patient Characteristics

The overall consistency of the EGFR mutation statuses in the 111 paired plasma and tissue samples was 71.2%. The sensitivity and specificity were 35.6 and 95.5%, respectively. We analyzed the consistencies, sensitivities and specificities for gender, age, smoking history, disease stage, pathology and tumor differentiation. A high overall consistency was exhibited by the subgroups of patients with poorly differentiated tumors (91.2%, 95% CI 79–98%; $p = 0.0053$) and squamous cell lung cancer (91.4%, 95% CI 77–98%; $p = 0.0014$). The sensitivity was significantly different in the disease stage and pathological differentiation subgroups. Sensitivity was 10% (95% CI 1–32%) in early-stage patients and 56% (95% CI 35–76%) in advanced-stage patients ($p = 0.0014$). For patients with poorly differentiated tumors, the overall sensitivity was 77.8% (95% CI 40–97%), which was significantly higher than that for the patients with highly differentiated (20%, 95% CI 3–56%; $p = 0.0230$) and moderately differentiated tumors (19%, 95% CI 5–42%; $p = 0.0042$). Table 4 shows the subgroup consistency, sensitivity and specificity analyses.

Table 3. Correlation of EGFR mutations in paired tissue and plasma samples

Plasma	Tissue		Total
	+	–	
+	16	3	19 (17.1)
–	29	63	92 (82.9)
Total	45 (40.5)	66 (59.5)	111 (100)

Figures represent numbers (percentages).

Discussion

The development of a noninvasive method of gene screening in cancer patients shows promise. However, the mutation statuses in the plasma/serum are not always consistent with those in the tissues. The concordances between the EGFR mutation statuses in the tissues and the plasma/serum samples in previous studies have varied from 58 to 97% and are listed in table 5. We hypothesize that the selection of specific patients would optimize the consistency of the results.

In our study, the overall consistency was 71.2%. We analyzed the consistencies and sensitivities of various patient subgroups. The consistency was significantly higher in squamous cell cancer (91.4%; $p = 0.0014$) and the poorly differentiated NSCLC subgroups (91.2%; $p = 0.0053$). In the squamous cell subgroup, the high consistency was due to the low overall mutation rate (5.7%). Of the 35 patients with squamous cell lung cancer, only 2 harbored EGFR mutations, and they were only detected in the tissue samples but not in the plasma in both cases. Because the purpose of noninvasive EGFR mutation detection is to efficiently detect positive results, we analyzed the sensitivity of detection in subgroups that were based on patient characteristics. Remarkably, the sensitivity varied significantly according to the disease stage and pathological differentiation; it was 10% in the early stage, 56% in the advanced stage, 20% in the highly differentiated patients and 19% in the moderately differentiated subgroup but reached 77.8% in the poorly differentiated subgroup. It is reasonable to suggest that EGFR mutations that are observed in cfDNA are more consistent with those in the tissues of patients with advanced-stage and poorly differentiated NSCLC. A previous study showed that the proportion of tumor-derived cfDNA varies according to the state and size of the tumor [8]. In addition, poorly differentiated

Table 4. Correlation between EGFR mutations and clinical characteristics

	Total number (pairs)	Consistency	Sensitivity	Specificity	p ₁	p ₂
Gender						
Female	35	24/35 (68.6)	10/20 (50.0)	14/15 (93.3)	0.6815	0.0702
Male	76	55/76 (72.4)	6/25 (24.0)	49/51 (96.1)		
Age						
≥60 years	59	44/59 (74.6)	9/24 (37.5)	35/35 (100)	0.2933	0.7708
<60 years	52	35/42 (67.3)	7/21 (33.3)	28/31 (90.3)		
Smoking history						
Ever	57	43/57 (75.4)	5/17 (29.4)	38/40 (95.0)	0.3078	0.5023
Never	54	36/54 (66.7)	11/28 (39.3)	25/26 (96.2)		
Disease stage						
I–IIIA	51	32/51 (62.7)	2/20 (10.0)	30/31 (96.8)	0.0708	0.0014
IIIB–IV	60	47/60 (78.3)	14/25 (56.0)	33/35 (94.3)		
Pathology						
Squamous cell	35	32/35 (91.4)	0/2 (0)	32/33 (97.0)	0.0014	0.5313
Non-squamous cell	76	47/76 (61.8)	16/43 (37.2)	31/33 (93.9)		
Differentiation						
High	20	12/20 (60.0)	2/10 (20.0)	10/10 (100)	0.0053	0.0044
Moderate	45	27/45 (60.0)	4/21 (19.0)	23/24 (95.8)		
Poor	34	31/34 (91.2)	7/9 (77.8)	24/25 (96.0)		
Undetermined	12	NA	NA	NA		
Total	111	79/111 (71.2)	16/45 (35.6)	63/66 (95.5)		

Figures represent numbers (percentage), except where indicated otherwise. NA = Not accessed; p₁ = p value of consistency; p₂ = p value of sensitivity.

Table 5. Review of studies assessing detection of EGFR mutations in serum or plasma

	n	Disease stage	Detection method		Consistency	Sensitivity	Specificity	Sample
			tissue	plasma/serum				
Bai et al. [20]	230	IIIB–IV	DHPLC	DHPLC	87%	82%	90%	plasma
Moran et al. [17]	121	IV	length analysis and Taqman	length analysis and Taqman	68%	NA	NA	serum
Present study	111	I–IV	ME-PCR	ME-PCR	71%	36%	96%	plasma
Goto et al. [21]	86	IIIB–IV	Scorpion-ARMS	Scorpion-ARMS	66%	43%	100%	serum
Ren et al. [18]	50	I–IIIA	direct sequencing	direct sequencing	70%	0	100%	serum
Kuang et al. [19]	43	IIIB–IV	direct sequencing	Scorpion-ARMS and DHPLC	74%	70%	85%	plasma
Kimura et al. [22]	42	IIIB–IV	direct sequencing	Scorpion-ARMS	93%	85%	94%	serum
Yung et al. [23]	35	III–IV	digital PCR	digital PCR	97%	92%	100%	plasma
Brevet et al. [24]	31	III–IV	RFLP	SMSG	61%	44%	85%	plasma
He et al. [25]	18	IIIB–IV	ME-PCR	ME-PCR	94%	100%	89%	plasma
Kimura et al. [26]	12	IIIB–IV	direct sequencing	direct sequencing	58%	66%	63/71% ¹	serum
Kimura et al. [9]	11	IIIB–IV	direct sequencing	Scorpion-ARMS	73%	50%	86%	serum

NA = Not assessed in original study; DHPLC = denaturing HPLC; Scorpion-ARMS = Scorpion-amplified refractory mutation system; RFLP = restriction fragment length polymorphism; SMSG = Sequenom mass spectrometry genotyping.

¹ Before/after treatment.

tumors are usually aggressive and have high potential for metastases. Therefore, the proportions of tumor-derived cfDNA may be higher in patients with advanced-stage and poorly differentiated NSCLC. A study assessing stage IV patients from Spain showed a high level of consistency (98.3%) in a patient with a performance status (PS) of 2 [17]. We did not analyze the relationship of the consistency or sensitivity with PS in this study, because a majority of the patients were classified as PS 0–1.

In subgroups that showed inconsistencies, the mutations were detected in either the tissues or plasma. Many hypotheses and explanations for this have been suggested. The large quantity of cfDNA that is derived from non-cancerous tissues may result in false negatives in the plasma/serum [8]. Taking the small proportion of cancer-derived cfDNA into account, highly sensitive methods, such as ME-PCR, a Scorpion-amplified refractory mutation system and denaturing HPLC may be applied. In this study, ME-PCR and sequencing were used to detect the EGFR mutations. However, although the detection sensitivity of ME-PCR was as high as 0.2%, only 40.5% (16/45) of the tumor-derived mutant DNA could be detected in the plasma. Notably, several previous studies have found that EGFR mutations were present in the plasma but not in tissues. In our study, we found EGFR mutations in 3 plasma samples but not in the paired tissues. For these tissue samples, 1, which was from a metastatic lymph node biopsy, contained a significant portion of lymphocytes that may have interfered with genotyping. The other 2 were obtained from transbronchial biopsies, which may not have provided sufficient material to elucidate the complete genetic makeup of the tumors. Therefore, for these 3 patients, it is possible that the tumor sequencing results were false negatives.

Of the 51 early-stage patients, EGFR mutations were present in 20 of the tissue samples but only in 3 of the plasma samples. One of these 3 patients had phase IIIA poorly differentiated adenocarcinoma, and the other 2 were phase I patients, 1 of whom harbored moderately differentiated adenocarcinoma and the other poorly differentiated squamous cell lung cancer. Previously, only one study on EGFR mutations in the plasma/serum had utilized early-stage patients. In that study, EGFR mutations were detected in 15 of 50 tissue samples; however, no mutations were detected in the plasma samples [18].

The findings in this study support the hypothesis that the use of blood samples may be broadly applicable for tumors with somatic mutations, including EGFR, K-RAS, BRAF, HER2 and PIK3CA, for which targeted drugs are available or in development. Moreover, the molecular

characterization of peripheral blood may provide a strategy for the noninvasive serial monitoring of tumor genotypes during treatment, especially for the EGFR T790M mutation, which is a secondary mutation that is related to acquired resistance to EGFR-TKIs [19]. There are some limitations considering the present analysis as a retrospective study. Only 111 patients were enrolled in the study. The sample size, especially for the poor differentiation subgroup, is not large enough to achieve a more definite conclusion. If a prospective study is conducted, the need for adequate amounts of plasma/serum, suitable DNA isolation methods and adequate sample sizes should be taken into consideration.

In summary, the current study demonstrates that plasma DNA may be a viable alternative to tumor samples for the detection of EGFR mutations, and it is most effectively applied in patients with advanced-stage or poorly differentiated NSCLC. Therefore, our results are promising, although they require further evaluation, because they are indicative of the utility of blood for gene testing.

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Financial Disclosure and Conflicts of Interest

There are no conflicts of interest to disclose.

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