

# Limitations in the Use of Serum Epidermal Growth Factor Receptor Mutations as Prognostic Markers for Non-Small-Cell Lung Cancer

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## Key Words

Serum · Epidermal growth factor receptor · Non-small-cell lung cancer · Tyrosine kinase inhibitor

## Abstract

**Objectives:** In this study, we surveyed patients with advanced non-small-cell lung cancer (NSCLC) who were undergoing tyrosine kinase inhibitor (TKI)-targeted therapy. Our aim was to determine whether epidermal growth factor receptor (EGFR) mutations in serum circulating tumor (ct)DNA are useful prognostic markers for NSCLC. **Subjects and Methods:** Serum samples were collected from 300 patients with NSCLC that included adenocarcinoma (ADC, n = 155) and squamous cell carcinoma (SCC, n = 145). DNA was extracted from the sera for the nested polymerase chain reaction (PCR) amplification of EGFR exons 18, 19 and 21 mutations. Direct sequencing of the PCR products was carried out in an automated 3730 sequencer. **Results:** The EGFR exons 18, 19 and 21 were successfully detected in the serum samples of 300 NSCLC patients. No EGFR mutation was found in the blood samples regardless of the characteristics of gender, age, ADC and SCC status or smoking history. **Conclusion:** No mutations in EGFR exons 18, 19 or 21 were identified in the serum ctDNA of these advanced-stage NSCLC patients undergoing

TKI-targeted therapy. More studies are needed on the use of EGFR mutations in serum ctDNA as guidance for TKI-targeted therapy.

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## Introduction

Lung cancer is the most common fatal cancer in men and women worldwide and non-small-cell lung cancer (NSCLC) constitutes 80% of lung cancer cases [1]. NSCLC patients without treatment have a median survival of <5 months. Chemotherapy usually prolongs survival for <6 months, and is often associated with significant toxicity [2].

Recently, tumor markers have come to play an increasingly important role in the detection and management of cancer. Laboratory-based tests are potentially useful for predicting drug response or resistance and for monitoring therapy in advanced disease [3]. Epidermal growth factor receptor (EGFR) is detectable in approximately 80–85% of patients with primary NSCLC [4]. Activation of the EGFR pathway results in the initiation of cancer proliferation, and increases the potential for metastasis and neoangiogenesis. The EGFR tyrosine kinase has been

implicated in the initiation and progression of NSCLC [4]. Inhibition of EGFR kinase activities by EGFR tyrosine kinase inhibitors (TKIs) like gefitinib and erlotinib results in effective treatment for patients with NSCLC [5, 6]. Indeed, the IRESSA Pan-Asia Study (IPASS) clinical trials in Asia on advanced NSCLC have confirmed that EGFR-activating mutations are the main predictor of clinical outcome with TKI therapy [7–9]. The most common EGFR-activating mutations are in exon 19 (del19) and exon 21 (L858R), which are found in approximately 10% of Caucasian patients with NSCLC and in up to 50% of Asian patients [10]. Therefore, the presence of EGFR mutations in tumor tissue has important prognostic value and this can be used to guide the treatment of lung carcinoma [11]. Of note, in mainland China, adenocarcinoma (ADC) and not smoking were found to be independent predictors for EGFR mutations in exons 18, 19 and 21 [12].

Unfortunately, there are several disadvantages involved in the detection of EGFR mutations in tumor tissue. The solid biopsy procedure is invasive, cannot usually be repeated and is not tolerated at all by vulnerable lung cancer patients. Furthermore, while the tumor is often heterogenous, the biopsy provides only a small amount of tumor tissue that may not be a representative sample. The use of solid biopsy also does not allow ongoing monitoring of genetic changes or drug sensitivity [13]. Liquid biopsy for detecting circulating tumor cells or circulating tumor (ct)DNA, on the other hand, does not have these drawbacks. In particular, the presence of ctDNA in the blood serum allows repeatable detection of EGFR mutations in NSCLC patients and the possibility of targeted therapy, and the heterogeneity of the tumor is no longer an issue [13].

The number of studies evaluating the potential use of EGFR mutations in ctDNA from serum or plasma in lung cancer diagnosis and prognosis has increased steadily in the past decade [14]. Several groups have utilized plasma or serum samples to demonstrate a correlation between EGFR mutations in ctDNA and the DNA in tumor tissue ranging from 59 to 92.9% [11, 15–20]. Nevertheless, liquid biopsies of blood samples to detect EGFR mutations in ctDNA are currently not used in clinical practice [13].

Methods that have been used to detect somatic mutations of EGFR from DNA in the bloodstream of NSCLC patients include mass spectrometry genotyping [11], mutant-enriched polymerase chain reaction (PCR) [21], microfluidics digital PCR [22], peptide nucleic acid-locked nucleic acid PCR and nested PCR. Each of these methods has its advantages [13]. However, mass spectrometry ge-

**Table 1.** Characteristics of 300 patients

	ADC		SCC	
	men	women	men	women
Subjects, n	82 (27.3)	73 (24.3)	135 (45)	10 (3.4)
Age, years				
Mean	56.3	55.0	60.2	55.1
Range	36–79	30–77	45–85	42–75
Age group, n				
<65 years	62 (20.7)	60 (20.0)	96 (32.0)	8 (2.66)
≥65 years	20 (6.7)	13 (4.3)	39 (13.0)	2 (0.7)
Smokers, n	62 (20.7)	3 (1.0)	103 (34.3)	0 (0)
Smoking history, n				
Never	20 (6.7)	70 (23.3)	32 (10.6)	10 (3.4)
~10 years	4 (1.4)	1 (0.3)	3 (1.0)	103 (34.3)
~20 years	6 (2.0)	1 (0.3)	23 (7.7)	0 (0)
~30 years	30 (10.0)	0 (0)	40 (13.3)	0 (0)
~40 years	18 (6.0)	1 (0.3)	29 (9.7)	0 (0)
~50 years	3 (1.0)	0 (0)	8 (2.6)	0 (0)
~60 years	1 (0.3)	0 (0)	0 (0)	0 (0)

Figures in parentheses are percentages.

notyping is time-consuming and costly, and mutant-enriched PCR is vulnerable to polymerase-induced errors and requires stringent reaction conditions. Microfluidics digital PCR and peptide nucleic acid-locked nucleic acid PCR are both costly, in terms of instrumentation and synthesis, respectively. Nested PCR, however, compared to these real-time PCR-based methods and even to next-generation sequencing technologies, generates little or no background amplification, has high sensitivity [23] and requires less time and expense [13]. In this study, we wanted to determine the feasibility of using ctDNA with EGFR mutations in exons 18, 19 and 21 as prognostic markers in NSCLC in the direct sequencing of nested PCR products, in order to assay the serum ctDNA from 300 advanced NSCLC patients undergoing TKI therapy.

## Subjects and Methods

### Patient Characteristics

Three hundred patients (217 men and 83 women) with advanced NSCLC examined and treated at the Zhejiang Cancer Hospital, Hangzhou City, China, from January to December 2012, were enrolled in this study. Clinical information, including gender, age, tumor pathology and smoking history, was available for all patients. The diagnosis of NSCLC was histopathologically or cytopathologically confirmed. The patients' characteristics are listed in table 1. Of the 300 patients, 155 (82 men and 73 women) had ADC, and 145 (135 men and 10 women) had squamous cell carcinoma

**Table 2.** Primer sequences

Primer	Sequences		Product length, bp	Sequencing end
	forward	reverse		
EGFR-E18	5'-ATGTCTGGCACTGCTTTC-3'	5'-CTCACAGGACCACTGATTAC-3'	395	R end
EGFR-E19	5'-CCCTCACCTTCGGGGTGCAT-3'	5'-CTCCAGGCTCACCAAGAGCA-3'	5,905	F end
EGFR-E21	5'-TCAAGCCCAGGTCTCAACT-3'	5'-CATTCCTGTCCCAGCAAG-3'	365	F end

**Table 3.** EGFR exon sequences

Exon	Size, bp	Amplification sequence
18	123	CTT GTG GAG CCT CTT ACA CCC AGT GGA GAA GCT CCC AAC CAA GCT CTC TTG AGG ATC TTG AAG GAA ACT GAA TTC AAA AAG ATC AAA GTG CTG GGC TCC GGT GCG TTC GGC ACG GTG TAT AAG
19	99	GGA CTC TGG ATC CCA GAA GGT GAG AAA GTT AAA ATT CCC GTC GCT ATC AAG GAA TTA AGA GAA GCA ACA TCT CCG AAA GCC AAC AAG GAA ATC CTC GAT
21	156	GGC ATG AAC TAC TTG GAG GAC CGT CGC TTG GTG CAC CGC GAC CTG GCA GCC AGG AAC GTA CTG GTG AAA ACA CCG CAG CAT GTC AAG ATC ACA GAT TTT GGG CTG GCC AAA CTG CTG GGT GCG GAA GAG AAA GAA TAC CAT GCA GAA GGA GGC AAA

(SCC). One hundred and sixty-eight were former or current smokers, including 3 women (all of whom had ADC), and the other 132 (44%) had never smoked. All patients accepted 1–4 cycles of platinum-based combined chemotherapy. All enrolled patients had no history of previous primary cancer other than lung cancer. All subjects provided their written informed consent, and the Ethics Committee of Zhejiang Cancer Hospital approved the study.

#### Blood Collection and DNA Extraction

Peripheral blood samples were obtained from each of the 300 patients in the course of treatment. The blood samples were immediately centrifuged at 1,600 g and 4°C for 10 min. The sera were transferred to new Eppendorf tubes and centrifuged once again at 160,000 g for 10 min. DNA was isolated from the serum of these patients using the Axyprep blood genomic DNA miniprep kit (Axygen Biosciences, Union City, Calif., USA). The extraction was performed according to the manufacturer's instructions. The extracted DNA was eluted in 50 µl buffer (provided by the kit) and stored at -20°C until used.

#### Nested PCR Amplification and Purification

Amplification of exons 18, 19 and 21 was done in duplicate for each serum sample. Primer sequences for EGFR exons 18, 19 and 21 nested PCR and extensions were designed by the Assay Designers software v3.0 (Sequenom) and processed according to standard protocols for iPLEX chemistry. Primers were synthesized by Sangon Biotech (Shanghai, China; table 2).

The first PCR assays were carried out in a volume of 50 µl containing 1 µl of extracted DNA, 1 µl of primers R1 and F1 and 0.5 µl

of Taq DNA polymerase. DNA was amplified for 35 cycles at 95°C for 3 min, 94°C for 30 s, 55–60°C for 35 s and 72°C for 40–50 s, followed by 5–8 min of repaired extension at 72°C.

The second set of PCRs was carried out in a volume of 25 µl that included 2 µl DNA from the first PCR products, 0.3 µl of primers R1 and F1 and 0.3 µl of Taq DNA polymerase. DNA was amplified at 95°C for 5 min, the next 35 cycles at 95°C for 30 s, 55°C for 35 s and 72°C for 30 s, and then a 10-min extension at 72°C. The PCR-amplified fragments of exons 18, 19 and 21 are 395, 590 and 365 bp, respectively. Nested PCR products were electrophoresed in 1% agarose gel, and only those that produced a positive band were purified using a PCR product purification kit (Sanprep, SK1141).

After nested PCR amplification and purification, all PCR products underwent bidirectional sequencing on ABI 3730 sequencers by means of ABI BigDye Terminator 3.1 chemistry.

#### EGFR Nested PCR Amplification Sequencing Analysis

Before sequencing, a PCR sequencing reaction for the PCR products of EGFR exons 18, 19 and 21 was performed using a BigDye Terminator v1.1 kit (Applied Biosystems, Foster City, Calif., USA) according to the kit instructions. All PCR assays were carried out in a volume of 20 µl that included 1 µl of purified PCR products, 8 µl of BigDye (2.5×) and 1 µM of primers (3.2 pmol/µl). The PCR sequencing reaction was performed at 96°C for 1 min, and then 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Sequencing was carried out in an ABI 3730 genetic analyzer (Applied Biosystems). All sequence variants were confirmed by sequencing the products of independent PCR amplifications.

### Statistical Analysis

EGFR mutation was not found in any of the samples from patients with advanced NSCLC, so statistical analysis was not performed.

## Results

### Patient Characteristics

The mean age of the patients with ADC and SCC was  $56.30 \pm 10.65$  years (range 30–79) and  $60.24 \pm 7.65$  years (range 42–85), respectively. EGFR exons 18, 19 and 21 were successfully detected in the serum samples of 300 NSCLC patients. The results showed that no EGFR mutation was found in the blood samples regardless of gender, age, ADC and SCC status or smoking history (table 3).

## Discussion

In this study, no mutation in the EGFR exons of the patients with advanced-stage NSCLC was found, which suggests that, consistent with previous studies [23, 24], the decision to conduct TKI therapy based on EGFR mutations in patient serum ctDNA is questionable. In fact, previous studies using liquid biopsies from NSCLC patients to detect EGFR mutations indicated that patient serum plays a limited role in the detection of EGFR mutations when compared to blood plasma [23] and pleural effusion [25]. In addition, a recent meta-analysis of 13 studies encompassing 1,591 NSCLC patients also indicated that serum DNA might not be reliable for detecting tumor EGFR mutations to guide EGFR-TKI treatment [24]. However, no EGFR mutation was found in tumor tissue-paired serum ctDNA from 50 resectable patients, and EGFR mutation was detected only in 2 serum ctDNA samples from 33 advanced-stage NSCLC patients [26]. The controversy over the feasibility of detecting EGFR mutations in serum ctDNA in NSCLC patients remains, because of a high correlation (87.7%) between the mutations in tumor tissue and tumor tissue-paired serum samples [20].

Although we found no mutation in EGFR exons 18, 19 or 21 in serum ctDNA, this does not mean that these EGFR mutations did not exist in any of the 300 NSCLC patients. EGFR mutations have been found to mainly occur in Asians, ADC patients and female patients who have never smoked. The EGFR-activating mutations exon 19 (del19) and exon 21 (L858R) account for up to 90% of all EGFR mutations [12], and can be found in up to 50% of Asian

NSCLC patients [21]. One likely reason for the failure to detect EGFR mutations in serum ctDNA in this study was the small sample population. Although there were 300 advanced-stage NSCLC patients, consisting of 155 ADC and 145 SCC cases, only 70 female NSCLC patients who did not have a smoking history had histopathologically diagnosed ADC. Therefore, further study with a larger population of patients may more reliably determine the possibility of detecting EGFR mutations in serum ctDNA.

In addition, serum ctDNA could be mostly from renewed normal cells [27], when primary or metastatic tumor mass cell necrosis or lysis occurs. This is especially the situation after platinum-based combined chemotherapy, in which many lung carcinoma cells from primary or metastatic sources are killed. Thus, the predominant nontumor DNA available after chemotherapy affects the detection of EGFR mutations in the sera of advanced ADC patients [28]. In this study, all enrolled patients had received 1–4 cycles of platinum-based combination chemotherapy. Therefore, a low percentage of mutated DNA within the entire ctDNA can lead to some false-negative results [13]. However, direct sequencing is usually used in the early detection of mutant DNA sequences in >30% of the total DNA sequences, and is less sensitive than the scorpion amplification refractory mutation system [22]. Further study to investigate EGFR mutations in serum ctDNA with a more sensitive detection technique is necessary, prepared both before and after platinum-based combined chemotherapy.

## Conclusion

No mutation in EGFR exons 18, 19 or 21 was detected in serum ctDNA from the patients with advanced-stage NSCLC who had received platinum-based chemotherapy. Our failure to detect the EGFR mutations in serum ctDNA warrants further endeavor in the application of serum ctDNA for the detection of EGFR mutations.

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