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ORIGINAL ARTICLE

Correlation between progression-free survival, tumor burden, and circulating tumor DNA in the initial diagnosis of advanced-stage *EGFR*-mutated non-small cell lung cancer

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Keywords
Circulating tumor DNA; EGFR-tyrosine kinase inhibitor (TKI); non-small cell lung cancer; progression-free survival; sensitivity.

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Abstract

Background: This study was conducted to identify whether the presence of circulating tumor DNA (ctDNA) in plasma before treatment with EGFR-tyrosine kinase inhibitors (TKIs) is associated with clinical outcomes.

Methods: Fifty-seven pairs of tissues and plasma samples were obtained from patients with NSCLC adenocarcinoma harboring activating *EGFR* mutations before the administration of EGFR-TKI treatment. ctDNA mutation was identified using the PANAMutyper EGFR mutation kit. Both qualitative and quantitative analyzes of the data were performed.

Results: Concordance rates with tissue biopsy were 40.4% and 59.6% for the qualitative and quantitative methods, respectively. Bone metastasis showed a statistically significant correlation with ctDNA detection (odds ratio 3.985, 95% confidence interval [CI] 1.027–15.457; $P = 0.046$). Progression-free survival (PFS) was significantly shorter in the group detected with ctDNA than in the undetected ctDNA group (median PFS 9.8 vs. 20.7 months; hazard ratio [HR] 2.30, 95% CI 1.202–4.385; $P = 0.012$). Detection of ctDNA before treatment with EGFR-TKIs (HR 2.388, 95% CI 1.138–5.014; $P = 0.021$) and extra-thoracic lymph node metastasis (HR 13.533, 95% CI 2.474–68.747; $P = 0.002$) were independently associated with PFS. Six of 11 patients (45.5%) monitored by serial sampling showed a dynamic change in ctDNA prior to disease progression.

Conclusion: Quantitative testing can increase the sensitivity of the ctDNA detection test. Patients with detectable ctDNA had significantly shorter PFS after receiving EGFR-TKIs than those with undetectable ctDNA. Tumor burden may be associated with plasma ctDNA detection. A shorter PFS was associated with detection of ctDNA and extra-thoracic lymph node metastasis. Dynamic changes in the ctDNA level may help predict clinical outcomes.

Introduction

Current practice for the treatment of patients with advanced-stage non-small cell lung cancer (NSCLC) has become increasingly dependent on identifying the presence of genetic mutations.[1–3] *EGFR* mutations can be detected in tissues extracted through surgery or other invasive procedures. In clinical situations, diagnosis and mutation testing should be performed simultaneously with tissue samples or cytology. However, a significant number of patients do not have enough tissue,[4] do not have a lesion available for

biopsy, or refuse to undergo a repeat biopsy. Complications from intrathoracic biopsy have been reported in approximately 17% of patients.[5] Because of the heterogeneity of NSCLC, a biopsy may need to be performed at multiple sites, which is frequently not realistic given the poor performance status of advanced-stage lung cancer patients.[6–10]

Given these limitations, alternative methods for analyzing the *EGFR* mutation status of NSCLC have been investigated. Non-invasive *EGFR* mutation detection methods based on plasma or serum show great potential. Circulating tumor DNA (ctDNA) in the plasma can be used to detect *EGFR* mutations in NSCLC patients, providing a level of information similar to that of tumor tissue biopsies.[11] Dynamic changes in ctDNA *EGFR* mutation status are associated with clinical outcomes of EGFR-tyrosine kinase inhibitor (EGFR-TKI) treatment, such as disease progression and progression-free survival (PFS).[12,13]

This study aimed to measure whether the presence of ctDNA in plasma before treatment with EGFR-tyrosine kinase inhibitors (TKIs) is associated with PFS. A qualitative and quantitative analysis was performed on the same samples and the results were compared.

Methods

Patients

Fifty-seven patients with NSCLC adenocarcinoma harboring activating *EGFR* mutations (exon 19 deletion and L858R mutation) at the Asan Medical Center, Seoul, Korea between January 2014 and December 2016 were enrolled in the study. E19 deletions and L858R mutations were detected in all patients via tissue biopsy and treated with EGFR-TKIs. Plasma samples were collected from patients before treatment with first-generation EGFR-TKIs (IRB No. 2016-0692).

Patient data were collected from medical records and radiological images to assess baseline information, tumor response, and PFS. Never smokers were defined as having smoked < 100 cigarettes in their lifetime. PFS was measured from the first day of treatment with EGFR-TKIs until tumor progression or the last follow-up date.

EGFR mutation analysis for tissue and plasma

We used direct Sanger sequencing or peptide nucleic acid (PNA)-mediated PCR clamping assay (PNAClamp EGFR Mutation Detection kit, PANAGENE Inc., Daejeon, Korea) to detect *EGFR* mutations in tissue. The PNA clamping probe binds to wild-type DNA and suppresses amplification. Only mutant-type DNA is selectively amplified.

The PANAMutyper EGFR kit (PANAGENE Inc.) was used to detect mutations in plasma. PANAMutyper is a technology that integrates PNAClamp and PANA RealTyper. PANA RealTyper is Multiplex Melting Curve Analysis using a fluorescence-labeled PNA probe. Fluorescent-labeled PNA probes only fluoresce when bound to the target sequence. PNA probes with a unique melting temperature (Tm) according to the nucleotide sequence are denatured from the target DNA at the Tm as the temperature increases and the fluorescence signal decreases. Genotyping of the target DNA is possible by analyzing the temperature at which the signal decreases (Tm). This method could increase sensitivity and allow the detection of ctDNA in plasma. Currently, the PANAMutyper EGFR kit is provided as a qualitative assay, but in this study, quantitative analysis was performed in the same manner. Quantitative results were converted into reactions according to the linearity equation and provided as semi-quantitative indices. The linearity of PANAMutyper R EGFR was evaluated by serial dilution of mutant *EGFR* standard materials spiked with human plasma wild type for *EGFR*. With this method < 1 copies/reaction and > 1000 copies/reaction are displayed as 1 and > 1000, respectively. The specificity of liquid biopsy for most ctDNA detection methods is close to 100%, and according to data provided by the laboratory, the specificity of the method used for ctDNA detection in this study was also 100%.

Statistical analysis

Fisher's exact and Wilcoxon rank-sum tests were used for categorical and continuous variables. The concordance rate was assessed by comparing the match between primary tissue and ctDNA sequencing results. Multiple logistic regression analysis was used to analyze risk factors associated with ctDNA detection and disease progression. The Kaplan–Meier method was used to estimate PFS distribution, and the Cox proportional hazards model was used to investigate the effect of specified risk factors on disease progression. Results were considered statistically significant at a P value of < 0.05. All analyses were performed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA).

Ethics statement

The Asan Medical Center institutional review board approved the study (No. 2016-1228). We received informed consent from all patients for study participation and genetic analysis.

Results

Patient characteristics

Baseline examination of *EGFR* mutations was performed in all patients using matched tumor tissues and plasma

Table 1 Baseline patient and tumor characteristics

Characteristics	All patients (n = 57)
Age (years) (mean ± SD)	57 ± 10.6
Gender, female (%)	39 (68.4)
Smoking status	
Never	41 (71.9)
Ever	16 (28.1)
ECOG performance status	
0	6 (10.5)
1	49 (86)
2	2 (3.5)
EGFR mutation by tissue biopsy	
Exon 19 del	39 (68.4)
L858R	18 (31.6)
Stage	
M0/< M1a	23 (40.4)
M1b	34 (59.6)
TKI	
Gefitinib	55 (96.5)
Erlotinib	1 (1.8)
Afatinib	1 (1.8)
Best response	
Partial response	44 (77.2)
Stable disease	12 (21.1)
Progressive disease	1 (1.8)

Unless otherwise stated, data are presented as number (%).ECOG, Eastern Cooperative Oncology Group; SD, standard deviation; TKI, tyrosine kinase inhibitor.

samples prior to treatment with first-generation EGFR-TKIs. The majority of patients were women (39/57, 68.4%), never smokers (41/57, 71.9%), and had extrathoracic metastatic diseases (M1b) (34/57, 59.6%). Most patients received gefitinib (55/57 96.5%) as first-line treatment. The most common mutation identified was an E19 deletion; T790M was not detected before treatment. Treatment responses were observed in 98.2% of patients, including partial responses (44/57, 77.2%) and stable disease (12/57, 21.1%) (Table 1). The median PFS was 14.9 months (95% confidence interval [CI] 7.55–22.25). During a median follow-up period of 23.3 months (interquartile range 14.3–29.0), disease progression and death occurred in 40 (70.2%) and 9 (15.8%) patients, respectively. All deaths were related to disease progression.

The two ctDNA *EGFR* mutation testing methods were compared for concordance with the tumor tissue. The concordance rates were 40.4% and 59.6% for the qualitative and quantitative methods, respectively (Table 2). Individual patient results are reported in Table S1. Receiver operating characteristic curve analysis was performed by quantitative test to predict the concordance rate between the qualitative analysis and tissue results. The area under the receiver operating characteristic curve was 0.804 (95% CI 0.686–0.922; $P < 0.001$) and the cutoff value was 1.5 copies/reaction (sensitivity 68.2%, specificity 71.4%). When the samples were dichotomized based on the cutoff value, the concordance rates between ctDNA qualitative analysis and the tissue results were 28.1% in < 1.5 copies/reaction and 100% in ≥ 1.5 copies/reaction.

At the time of diagnosis, there was a statistically significant correlation between bone metastasis and ctDNA detection (Table 3). The ability to detect ctDNA using the qualitative test did not have a statistically significant effect on PFS (median PFS: ctDNA detected 11.5 vs. ctDNA undetected group 13.5 months, hazard ratio [HR] 1.417, 95% CI 0.80–2.52; $P = 0.234$) (Fig 1a). However, in the quantitative test results, PFS was statistically significantly shorter in the ctDNA detected than in the ctDNA undetected group (median PFS 9.8 vs. 20.7 months, respectively, HR 2.30, 95% CI 1.202–4.385; $P = 0.012$) (Fig 1b). Detection of ctDNA before treatment with EGFR-TKIs (HR 2.388, 95% CI 1.138–5.014) and extrathoracic lymph node (LN) metastasis (HR 13.533, 95% CI 2.474–68.747) were independently associated with PFS (Table 4).

Dynamic changes in *EGFR* mutations were analyzed in 11 patients using serial sampling. In four patients, ctDNA mutations were unable to be detected from diagnosis to disease progression. In one patient, there was no change in the level of ctDNA. Dynamic changes in the ctDNA level were observed in the remaining six patients (Fig 2). Negative conversion of detectable ctDNA after EGFR-TKI administration was found in 45.5% (5/11) of patients

Table 2 Comparison of plasma and tissue samples according to qualitative (PANAMutyper) and quantitative (PANAgene-SQI) ctDNA tests

		Tissue *EGFR* mutation		
Testing method		E19del	L858R	Total patients
ctDNA *EGFR* mutation	E19del	14 (35.9%)	0	14
(PANAMutyper)	L858R	0	9 (50%)	9
	Wild	25	9	34
	Total patients	39	18	57
ctDNA *EGFR* mutation	E19del	21 (53.8%)	(1)	21
(PANAgene-SQI)	L858R	0	13 (72.2%)	13
	Wild	18	5	23
	Total patients	39	18	57

E19del, exon 19 deletion; ctDNA, circulating tumor DNA.

Table 3 Multivariate analysis of risk factors for ctDNA detection

Variable	Multivariable analysis	
	OR (95% CI)	*P*
Age	6.182 (0.481–79.444)	0.691
Gender (male)	7.291 (0.734–72.435)	0.090
Smoking	5.928 (0.552–63.367)	0.142
Metastasis to pleural effusion	0.657 (0.085–5.077)	0.469
Pericardial metastasis	0.227 (0.0–241.538)	0.955
Metastatic pleural nodules	0.648 (0.100–4.189)	0.833
Contralateral lung metastasis	1.216 (0.225–6.567)	0.598
Bone metastasis	3.985 (1.027–15.457)	0.046
Extrathoracic lymph nodes metastasis	11.533 (2.200–60.469)	0.257
Brain metastasis	0.448 (0.096–2.490)	0.467
Adrenal metastasis	7.482 (0.363–154.317)	0.125
Liver metastasis	—	0.999

CI, confidence interval; ctDNA, circulating tumor DNA; OR, odds ratio.

(Fig 2b–f), and increased ctDNA levels before or after disease progression were observed in 54.5% (6/11). T790M mutation was detected in 36.4% (4/11) of patients before or after disease progression (Fig 2c–f).

Discussion

Although ctDNA is now actively studied, numerous challenges need to be overcome before ctDNA can be used as a routine method of detection in a clinical setting. Although the specificity of liquid biopsy is close to 100% regardless of the technique used, the sensitivity is lower and varies by the method used.[13–16] When very few copies of ctDNA are available, it is not always detectable in the peripheral blood, thus ctDNA analysis results might be false negative. Therefore, one major challenge is low

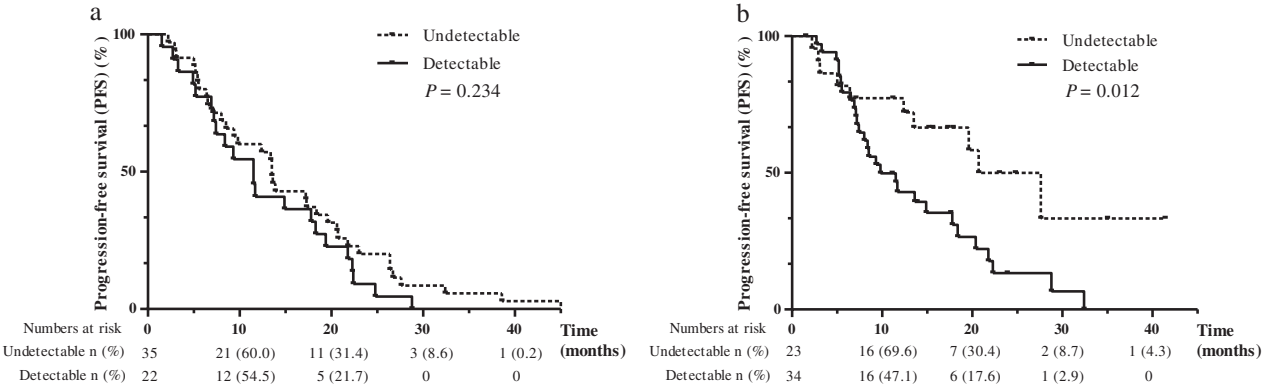

Figure 1 Kaplan–Meier survival curves of progression-free survival of patients to circulating tumor DNA (ctDNA) *EGFR* mutation. (**a**) Qualitative and (**b**) quantitative analysis of ctDNA.

Table 4 Univariate and multivariate analysis of prognostic factors for progression-free survival

Variable	Univariate analysis		Multivariable analysis	
	HR* (95% CI)	*P*	HR* (95% CI)	*P*
Age	0.978 (0.948–1.010)	0.174	0.970 (0.939–1.002)	0.070
Gender	0.797 (0.400–1.589)	0.520	0.881 (0.433–1.789)	0.774
ctDNA (+)	2.483 (1.196–5.156)	0.015	2.388 (1.138–5.014)	0.021
Smoking	1.452 (0.716–2.947)	0.314		
Histologic differentiation	2.981 (1.018–8.731)	0.088		
M1b metastasis	2.453 (1.159–5.194)	0.019	1.843 (0.836–4.063)	0.128
Metastasis to pleural effusion	0.854 (0.258–2.834)	0.797		
Pericardial metastasis	1.129 (0.152–8.358)	0.905		
Pleural nodules	0.290 (0.112–0.748)	0.010		
Contralateral lung metastasis	0.898 (0.423–1.906)	0.778		
Bone metastasis	1.769 (0.935–3.346)	0.080	0.761 (0.339–1.706)	0.783
Extrathoracic lymph nodes metastasis	10.731(2.258–51.004)	0.003	13.533 (2.474–68.747)	0.002
Brain metastasis	1.140 (0.593–2.194)	0.694		
Adrenal metastasis	2.155 (0.814–5.708)	0.122		
Liver metastasis	2.386 (0.822–6.923)	0.110		
Other sites (metastasis)	3.133 (0.414–23.730)	0.269		

CI, confidence interval; ctDNA, circulating tumor DNA; HR, hazard ratio.

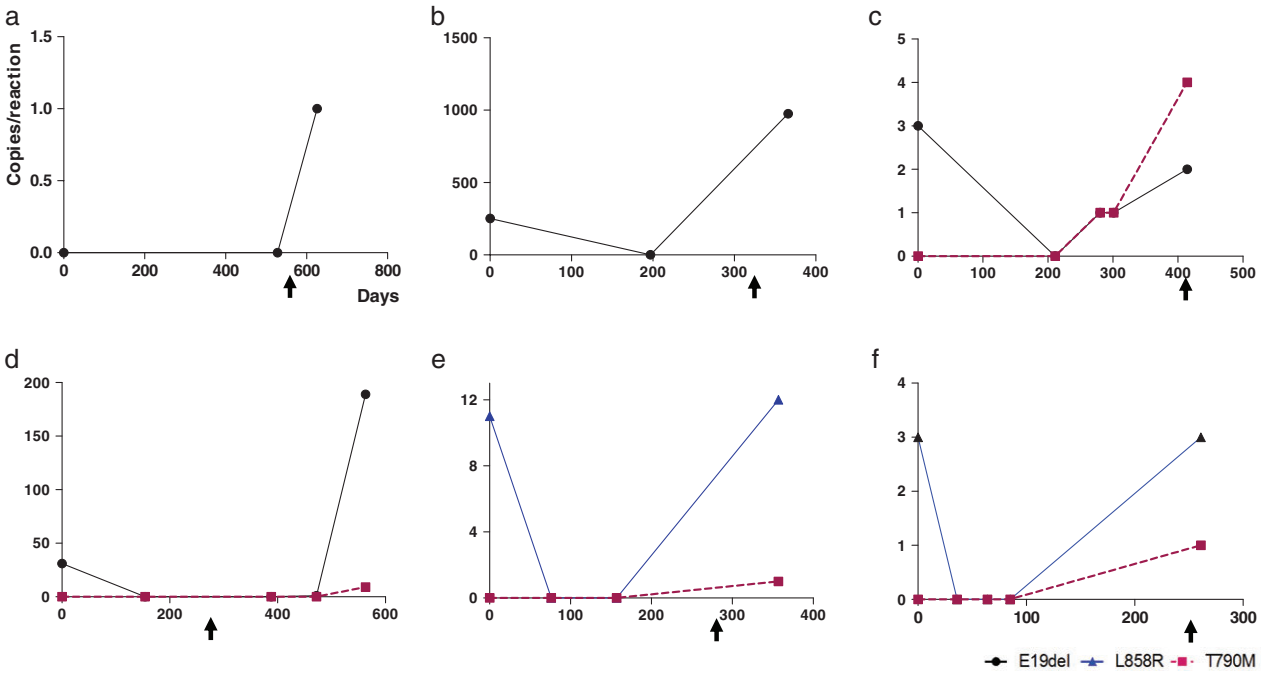

Figure 2 (**a–f**) Serial monitoring in six patients detected with circulating tumor DNA after EGFR-tyrosine kinase inhibitor administration. Arrows indicate disease progression.

sensitivity. The concordance rate of ctDNA depends on which method is used;[17–20] however, few studies have performed both quantitative and qualitative analysis of ctDNA using the same sample. In this study, the detection rate of ctDNA was higher by quantitative than by qualitative analysis. To analyze the cause of these differences, we individually identified all results of the registered patients (Table S1). When the data were divided into two groups according to the amount of ctDNA detected by quantitative analysis, the detection rate of ctDNA by the qualitative test was higher in the group with > 1.5 copies/reaction. This discrepancy may be attributable to several factors. Because pre-analysis conditions undoubtedly play a crucial role,[21] slight differences in plasma DNA extraction and quantification methods in each laboratory may affect the detection rate of ctDNA. Moreover, the detection rate could vary depending on the threshold setting.[22,23]

In current clinical practice, only qualitative analysis of ctDNA is performed. This study highlights the potential for sensitivity enhancement by using quantitative analysis, as we found that additional quantitative analysis can increase the sensitivity of the ctDNA detection test.

Several studies have shown that high ctDNA copy numbers are associated with shorter overall survival and PFS.[24–26] In our study, detection of ctDNA by qualitative analysis was not statistically significant to PFS, but detection of ctDNA by quantitative analysis yielded a statistically significantly shorter PFS. For these reasons, quantitative analysis of ctDNA may be more pertinent.

Although some studies have found no correlation between ctDNA levels and tumor burden,[24,27] most studies have suggested that detection of ctDNA is significantly associated with tumor burden.[28–31] In this study, bone metastasis was associated with detectable ctDNA, supporting the higher detection rate of ctDNA in patients with a more extensive tumor burden.

This study showed a pattern of decreased ctDNA levels after treatment that increased before or after tumor progression, which mirrored results found in other studies.[32,33] In one patient, increased ctDNA levels and T790M mutation appeared much earlier than tumor progression, as assessed by imaging (Fig 2c). These results indicate that quantitative analysis of ctDNA can be used as a marker of therapeutic response or the development of resistance. Although we achieved meaningful results, a limitation of this study was the small patient population.

An attractive feature of ctDNA is that it contains DNA mutations found in primary and metastatic lesions,[34–36] which may be a way to overcome problems caused by tumor heterogeneity. More studies should be performed to validate the potential of ctDNA for routine clinical use, and precise medicine is the next step in the treatment of NSCLC.

Quantitative testing can increase the sensitivity of ctDNA detection. Patients with detectable ctDNA had

significantly shorter PFS after receiving EGFR-TKI than patients with undetectable ctDNA. Tumor burden may be associated with plasma ctDNA. A shorter PFS was associated with detection of ctDNA and extrathoracic LN metastasis. Dynamic changes in the ctDNA level may help predict clinical outcomes.

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Disclosure

No authors report any conflict of interest.

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Supporting Information

Additional Supporting Informationmay be found in the online version of this article at the publisher's website:

Table S1 Comparison between qualitative and quantitative analysis of circulating tumor DNA (ctDNA) *EGFR* mutation.

