


ORIGINAL ARTICLE

Presence of mEGFR ctDNA predicts a poor clinical outcome in lung adenocarcinoma

Taehee Kim , Eun Young Kim, Sang Hoon Lee, Do Sun Kwon, Arum Kim & Yoon Soo Chang

Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Republic of Korea

Keywords

ctDNA; ddPCR; lung adenocarcinoma; mEGFR.

Correspondence

Dr Yoon Soo Chang, Department of Internal Medicine, 4th Floor, Research Center for Future Medicine, Yonsei University College of Medicine, 63-gil 20, Eonju-ro, Gangnam-gu, Seoul, Republic of Korea.

Tel: +82 2 2019 3309

Fax: +82 2 3463 3882

Email: yschang@yuhs.ac

Received: 13 August 2019;

Accepted: 25 September 2019.

doi: 10.1111/1759-7714.13219

Thoracic Cancer **10** (2019) 2267–2273**Abstract****Background:** Circulating tumor DNA (ctDNA) is a biomarker for the selection of target agents in various malignancies. In this study, we examined the effect of ctDNA presence on the response to EGFR-tyrosine kinase inhibitor (TKI) and on the prognosis in lung adenocarcinoma.**Methods:** ctDNA of EGFR-TKI sensitizing mutations (mEGFR), L858R substitution and Exon 19 deletion (E19d) mutation, was evaluated using droplet digital PCR (ddPCR) in 81 patients with lung adenocarcinoma which harbored mEGFR in the corresponding tumor tissues.**Results:** The study recruited lung cancer patients at various stages, and the sensitivity, specificity, and area under the curve (AUC) of mEGFR ctDNA detection by ddPCR were 40.0%, 88.5%, and 0.68, respectively. It showed higher sensitivity (75.0% vs. 10.0%) and AUC (0.83 vs. 0.49) in the advanced stages of lung adenocarcinoma compared with the early stages and the number of metastases and the fractional abundance of mEGFR ctDNA showed a strong correlation ($\sigma = 0.516$; $P < 0.001$, Spearman correlation test). There was a significantly shorter progression-free survival and duration of disease control by EGFR-TKIs in the ctDNA-positive group than the negative group (14.0 vs. 41.0 months, $P = 0.02$ and 12.0 vs. 23.0 months, $P = 0.02$, log-rank test, respectively). There was a trend for overall survival time to be shorter in patients with mEGFR ctDNA than for patients without mEGFR ctDNA (35.6 vs. 67.1 months, $P = 0.06$, log-rank test).**Conclusions:** These data showed that mEGFR ctDNA detection using ddPCR is useful in the advanced stages and its presence predicted distant metastasis and poor clinical outcome in lung adenocarcinoma.**Introduction**

In 2018, approximately two million people were diagnosed with lung cancer globally, accounting for 11.6% of the total cancer incidence and the death toll from this devastating disease was 1.76 million, accounting for 18.4% of cancer-related deaths, remaining the most common cause of cancer-related deaths to date.¹ A total of 85% of lung cancers are non-small cell lung cancer (NSCLC). More than 50% of NSCLC patients present with distant metastasis at the time of diagnosis, which cannot be cured by surgical resection alone.²

Drug-sensitizing mutations in the tyrosine kinase domain of the epidermal growth factor receptor (mEGFRs) occur frequently in lung adenocarcinomas of women, never smokers, and Asians. In Korea, mEGFRs are found in 40%

of lung adenocarcinomas.³ Large-scale phase III clinical trials have been performed in patients with mEGFR-positive lung adenocarcinoma and showed a higher response rate and prolonged progression-free survival (PFS) in the group treated with EGFR tyrosine kinase inhibitors (TKIs) compared to those treated with cytotoxic chemotherapy. When *EGFR* mutations were present in the tissues, the therapeutic response to EGFR-TKIs was more than 70%.^{4–8} As a result, EGFR-TKIs are widely used as the standard treatment option for mEGFR-positive lung adenocarcinoma.

When a small amount of specimen is collected, it may be inadequate to use for genetic testing, including testing for *EGFR* mutations. In this case, liquid biopsies can be used for adjunctive diagnostic testing. The detection of

circulating tumor DNA (ctDNA) is used for the selection of the most suitable diagnostic option. Currently, Cobas amplification refractory mutation system (ARMS, Cobas EGFR Mutation Test v2), next-generation sequencing (NGS)-based methods, peptide nucleic acid (PNA)-mediated PCR, and droplet digital PCR (ddPCR) methods can be used to detect ctDNA in liquid biopsies from NSCLC patients. Of these methods, Cobas II and PNA-mediated PCR are approved in the Republic of Korea.⁹

Recently, ddPCR, which is one of the most sensitive methods, has been used to detect *EGFR* mutant ctDNA in NSCLC patients.^{10–13} However, there are limited studies on the interpretation of ddPCR results, the cutoff values, and the clinical implication of these values. Therefore, the aim of this study was to determine the cutoff values and the clinical implications of ddPCR parameters measured in plasma samples.

Methods

Patients

We analyzed the results of patients as follows: (i) Had a pathologically confirmed lung adenocarcinoma; (ii) *EGFR* mutation status was confirmed in the tumor tissue; (iii) Computed tomography had been used to evaluate the primary lesion size; (iv) No previous therapy had been experienced before blood sampling; and (v) The patient agreed to provide blood samples. Both *EGFR* L858R substitution and exon 19 deletion (E19d) mutation tests were performed by ddPCR on collected blood samples.

All patients provided their written informed consent. This study was performed in accordance with the amended Declaration of Helsinki, following approval of the study by an independent hospital institutional review board (approval No. 3-2016-0225 and 3-2017-0321).

Plasma collection and DNA extraction

In eligible patients, plasma was collected using a Streck tube (Dow Biomedica, Seoul, Korea). The collected blood was centrifuged at $1000 \times g$ for 10 minutes within the same working day and the supernatant was separated and stored at -80°C until DNA extraction. ctDNA was extracted from the plasma using a QIAamp circulating nucleic acid kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

ddPCR

ddPCR was performed according to the manufacturer's manual (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Mutation sites were detected using probes for *EGFR* L858R (BR186dHsaCP2000021; Bio-Rad) and Exon19del

(BR12002392, Bio-Rad). ddPCR was performed by first forming droplets with a QX100 Droplet Generator (Bio-Rad). PCR amplification was then performed within each droplet using a thermal cycler (Bio-Rad). Following PCR, droplets were streamed in single file on a QX200 Droplet Reader (Bio-Rad), which counts the fluorescent-positive and fluorescent-negative droplets to calculate the target DNA concentration. Data were processed using QuantaSoft software (Bio-Rad). The threshold values of the ddPCR results were determined using QuantaSoft software and then manually validated. ddPCR results passed quality control when the number of droplets was more than 9000 and the wild-type gene sequence was present in more than 100 copies/mL. Only data that passed initial quality control were used for analysis.

ddPCR test results yielded three parameters: concentration, mutant/wild-type ratio, and fractional abundance. Area under the curve (AUC) values were calculated to determine which of the three parameters were most consistent with that from tumor tissue. For E19d results, the AUC values were 0.67 for the concentration, the mutant/wild type ratio, and fractional abundance. The AUC values for the L858R results were 0.66 for the concentration, the mutant/wild-type ratio, and fractional abundance. There were no significant differences in these values and the commonly used fractional abundance parameter was used for further analysis. Results were defined as positive when the fractional abundance was greater than 0.1%.

Statistical analysis

Categorical and continuous parameters were evaluated using a Chi-square test or an independent sample *t*-test, respectively. The Spearman correlation test was used to test relationships between two variables. Survival curves were generated using the Kaplan-Meier method. *P*-values less than 0.05 were considered significant. Statistical analyses were performed using SPSS version 23.0 for Windows (SPSS, Chicago, IL, USA) or R Statistical Package (Institute for Statistics and Mathematics, Vienna, Austria, ver 3.5.3, www.R-project.org).

Results

Clinical characteristics of study population

To confirm the robustness of ddPCR testing parameters and determine the appropriate cutoff value, 81 patients who had been diagnosed with lung adenocarcinoma and underwent tissue *EGFR* mutation testing were recruited. The baseline characteristics of the patients are presented in Table 1. The mean age of the patients was 64.0 ± 1.1 years; there were 43 (53.1%) males, and 52 (64.2%) patients had no smoking

Table 1 Baseline characteristics of lung adenocarcinoma patients ($n = 81$)

Variables	Total (No.)	%
Mean age, years	64.0 ± 1.1	
Sex		
Male	43	53.1
Female	38	46.9
Smoking history		
Never smoker	52	64.2
Ex-smoker	22	27.2
Current smoker	7	8.6
T stage		
Primary tumor size, cm	3.67 ± 2.57	
T1	24	29.6
T2	23	28.4
T3	9	11.1
T4	25	30.9
N stage		
N0	39	48.1
N1	1	1.2
N2	19	23.5
N3	22	27.2
M stage		
Metastatic sites	0.73 ± 1.03	
M0	47	58.0
M1	34	42.0
Stage		
I	25	30.9
II	6	7.4
III	14	17.3
IV	36	44.4
EGFR mutation		
Wild	31	38.3
L858R	20	24.7
E19del	24	29.6
Others	6	7.4

history. The stage distribution in patients was as follows: stage I in 25 (30.9%), stage II in six (7.4%), stage III in 14 (17.3%), and stage IV in 36 (44.4%) patients. A total of 31 (38.3%) patients showed the wild-type EGFR genotype, 20 (24.7%) the L858R, and 24 (29.6%) the E19d mutant.

The receiver operating characteristic (ROC) curve analysis was then performed to evaluate the representativeness of plasma ctDNA on tumor tissue mutation status and AUC values were calculated. In this cohort comprised of heterogeneous stages, the AUC of plasma ddPCR analysis was 0.67 (95% CI, 0.56–0.77) for E19d and 0.66 (95% CI, 0.53–0.79) for L858R. Plasma ddPCR analysis showed high specificity for the detection of E19d (91.2%) and L858R (96.7%). The sensitivity of the plasma ddPCR method was modest for E19d (41.7%) and L858R (40.0%) (Figure S1a, d). The diagnostic applicability of the plasma ddPCR method for mEGFR detection was analyzed without discriminating between E19d and L858R (Fig 1a). As a result,

the AUC was 0.68 (95% CI, 0.58–0.77), the sensitivity was 40.0%, and the specificity was 88.5% for all stages.

ctDNA showed higher correspondence in advanced lung adenocarcinoma

We further analyzed the data according to the stage, with early stage defined as stages I–IIIA and advanced stage as stages IIIB–IV. In patients with early stage disease, the AUC of the plasma ddPCR analysis was 0.46 (95% CI, 0.41–0.51) for E19d (Figure S1b) and 0.51 (95% CI, 0.38–0.64) for L858R (Figure S1e). The sensitivity was 0.0% for E19d and 11.1% for L858R and the specificity was 92.3% for E19d and 100.0% for L858R. When the detection rates of L858R and E19d mutants were analyzed together in early-stage patients, the AUC was 0.49 (95% CI, 0.37–0.61), the sensitivity was 10.0%, and the specificity was 100.0% (Fig 1b). In the advanced stage patients, the AUC of the plasma ddPCR analysis was 0.84 (95% CI, 0.71–0.98) for E19d (Figure S1c) and 0.79 (95% CI, 0.62–0.96) for L858R (Figure S1f). The sensitivity was 76.9% for E19d and 63.6% for L858R and the specificity was 90.3% for E19d and 97.0% for L858R. When the detection rates of L858R and E19d mutants were analyzed together in the advanced-stage patients, the AUC was 0.83 (95% CI, 0.71–0.94), the sensitivity was 75.0%, and the specificity was 85.0% (Fig 1c). Taken together, the agreement between the plasma ddPCR results and that of the tissue biopsy genotype increased in advanced stage, suggesting the mEGFR detection by ddPCR is only applicable in the advanced stage.

Relationship of number of distant metastatic sites to presence of mEGFR ctDNA

To investigate the clinical factors associated with the presence of mEGFR ctDNA in the blood, a correlation analysis was performed between the clinical parameters and the fractional abundance from ddPCR analysis. First, the relationship between TNM stage and plasma ctDNA fractional abundance was analyzed (Table 2). As the TNM stage increased, the proportion of ctDNA *EGFR* mutation tended to increase. There was no significant difference in the fractional abundance value of mEGFR ctDNA with the progression of T stage and N stage (Figure S2). Regarding the M stage, there was a significant difference in the fractional abundance value in those with and without distant metastasis ($0.45 \pm 0.24\%$ in M0 and $8.71 \pm 3.14\%$ in M1, $P < 0.001$). In addition, the correlation between the number of metastatic sites and plasma ctDNA fractional abundance was analyzed, and there was a strong correlation ($\sigma = 0.516$, $P < 0.001$, Spearman correlation test, Fig 2d). The most common metastatic sites in stage IV patients were bone (44.4%, 16/36) and the brain (33.3%, 12/36). There was also a significant difference in the fractional

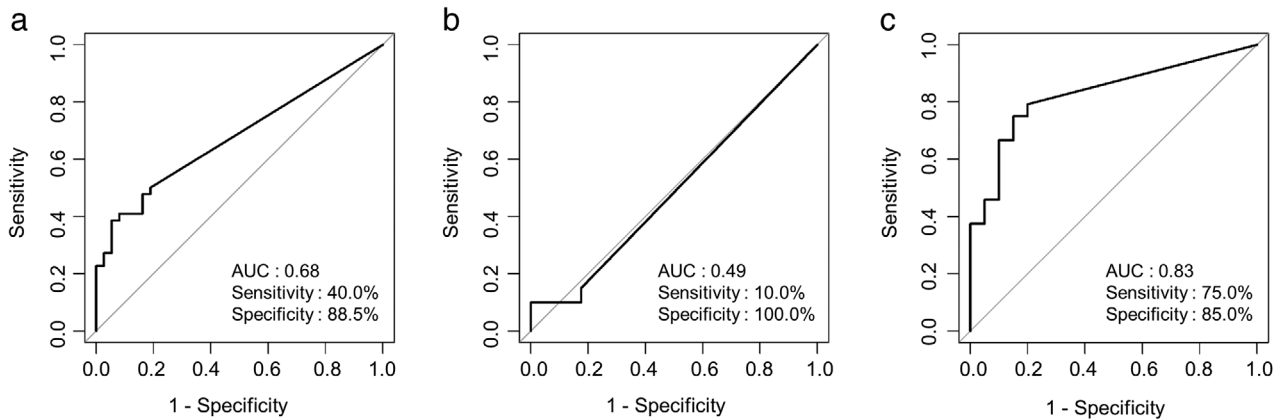


Figure 1 ROC analysis of plasma ddPCR assay of NSCLC tissue samples. (a) Overall stage, (b) Early stage (I–IIIa), and (c) Advanced stage (IIIb–IV).

abundance values in patients with or without bone metastasis (Fig 2b) and brain metastasis (Fig 2c). Therefore, the detection of plasma ctDNA is useful as a surrogate marker for predicting distant metastasis.

Presence of mEGFR ctDNA predicts a poor clinical outcome in lung adenocarcinoma

The prognostic significance of plasma ctDNA detection was analyzed in 81 lung adenocarcinoma patients. Of the 81 patients, 52 (64.2%) were mEGFR ctDNA negative and

29 (35.8%) were positive. The PFS and overall survival (OS) of the patients were analyzed divided to mEGFR ctDNA positive group and mEGFR ctDNA negative group (Fig 3). The median PFS of lung adenocarcinoma patients was 22.0 months (95% CI, 15.8–28.2 months). According to Kaplan-Meier survival curves, the ctDNA positive group had significantly shorter PFS than the ctDNA negative group (14.0 vs. 41.0 months, $P = 0.02$, log-rank test, Fig 3a). The mean OS was 58.3 ± 3.7 months (95% CI, 51.0–65.5 months). Although statistical significance was not reached, the OS of the ctDNA positive group was shorter

Table 2 The relationship between the parameters related to the TNM staging and plasma ctDNA fraction abundance

	N (%) 81(100)	Fraction abundance (%)	P-value	ctDNA EGFR mutation (%)	
T stage			0.001*		0.01***
T1	24 (29.6)	0.55 ± 0.52		3/24 (12.5)	
T2	23 (28.4)	2.63 ± 1.80		6/23 (26.1)	
T3	9 (11.1)	0.11 ± 0.11		1/9 (11.1)	
T4	25 (30.9)	3.76 ± 1.58		13/25(52.0)	
N stage			0.004*		0.013***
N0	39 (48.1)	0.52 ± 0.47		5/39 (12.8)	
N1	1 (1.2)	NA		0/1 (0)	
N2	19 (23.5)	2.82 ± 1.84		8/19 (42.1)	
N3	22 (27.2)	4.32 ± 1.95		10/22 (45.5)	
M stage			<0.001**		<0.001***
M0	47 (58.0)	0.69 ± 0.47		6/47 (12.8)	
M1	34 (42.0)	4.01 ± 1.57		17/34 (50.0)	
Stage			<0.001**		0.001***
I	25 (30.9)	0.15 ± 0.01		2/25 (8)	
II	6 (7.4)	0.02 ± 0.02		0/6 (0)	
III	14 (17.3)	0.96 ± 0.89		3/14 (21.4)	
IV	36 (44.4)	4.29 ± 1.54		18/36 (50.0)	
Brain metastasis			<0.001**		
No	69 (85.2)	1.41 ± 0.60			
Yes	12 (14.8)	5.98 ± 3.44			
Bone metastasis			<0.001**		
No	65 (80.2)	0.45 ± 0.24			
Yes	16 (19.8)	8.71 ± 3.14			

*Kruskal-Wallis test. **Mann-Whitney U test. ***Chi-square test.

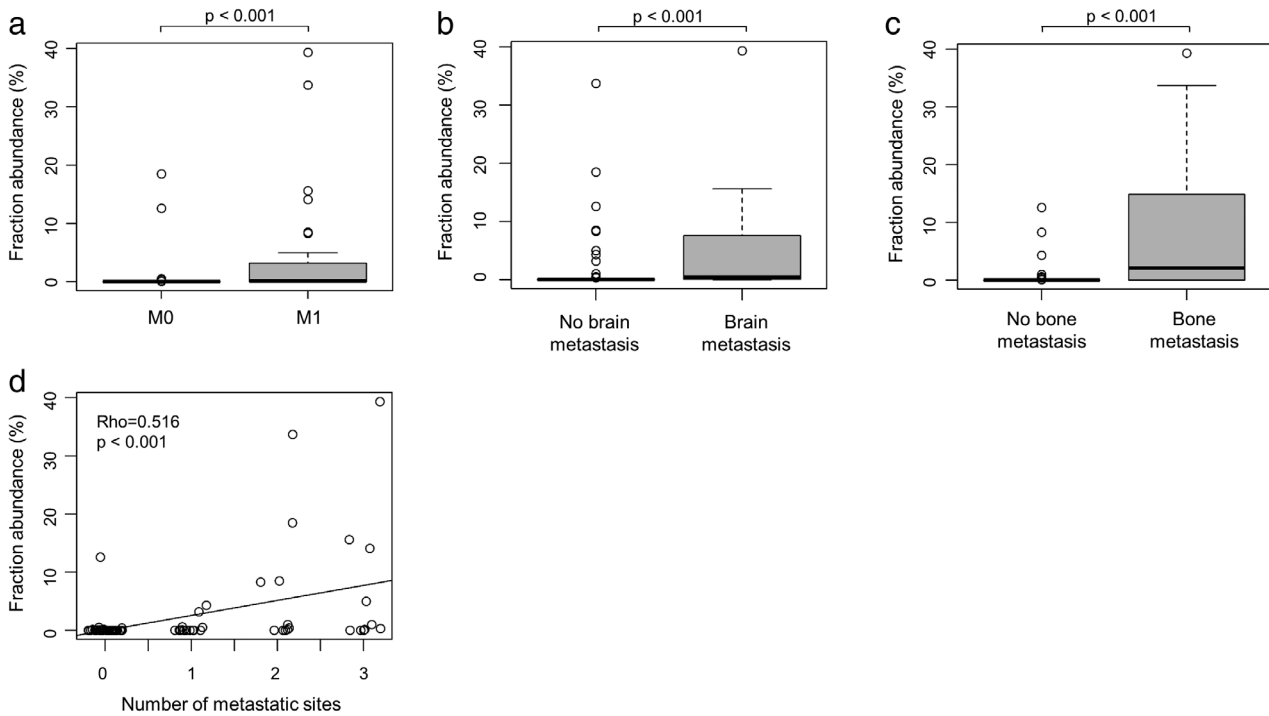


Figure 2 Correlation between ddPCR fractional abundance for EGFR mutations and clinical parameters: (a) M stage, (b) Brain metastasis, (c) Bone metastasis, and (d) the number of metastatic sites.

than that of the ctDNA negative group (35.6 ± 3.20 vs. 67.1 ± 1.88 months, $P = 0.06$, log-rank test, Fig 3b). A subgroup analysis was performed in 27 patients who had been diagnosed with mEGFR-positive lung adenocarcinoma and received EGFR-TKI treatment. Among the 26 patients, five (19.2%) were plasma ctDNA negative and 21 (80.8%) were plasma ctDNA positive. PFS after the initiation of EGFR-TKI treatment was analyzed in these two groups. The median PFS time of patients taking EGFR-TKIs was 13.0 months (95% CI: 8.86–17.1 months). The duration of disease control by EGFR-TKIs was 11 months shorter in the plasma ctDNA positive group than the

ctDNA negative group (12.0 vs. 23.0 months, $P = 0.02$, log-rank test, Fig 3c). In conclusion, the presence of mEGFR ctDNA was associated with poor survival in lung adenocarcinoma patients and poor treatment response to EGFR-TKI treatment.

Discussion

In this study, we demonstrated the high sensitivity and specificity of plasma ctDNA detection in NSCLC patients. The plasma ddPCR assay used in this study was similar to, or improved, compared with other ctDNA genotyping

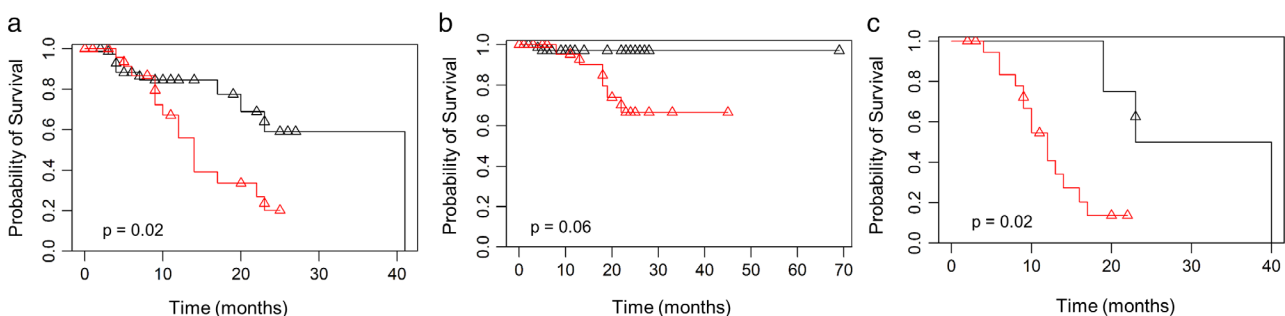


Figure 3 Survival analysis according to the presence of EGFR mutations in ctDNA. (a) PFS, (b) OS in lung adenocarcinoma ($n = 81$) and (c) PFS in lung adenocarcinoma patients treated with EGFR-TKIs ($n = 27$). (—) ctDNA negative, (—) ctDNA positive, (—△) Censored ctDNA negative, and (—△) Censored ctDNA positive.

assays.^{14–19} Previous studies have investigated the detection of plasma ctDNA only in patients with advanced NSCLC. In contrast, this study compared plasma ctDNA detection rates in early and advanced stages of NSCLC. These results showed that the plasma ddPCR assay was only useful in the advanced stage because it had relatively low diagnostic yields in the early stage. This study demonstrated the usefulness of a ddPCR-based plasma genotyping assay was dependent on the cancer stage.

Previous studies have reported correlations between plasma ctDNA and factors associated with TNM staging. Lee *et al.* reported that plasma ctDNA is not correlated with primary tumor size, but is significantly correlated with the number of metastatic sites.¹⁶ Couraud *et al.* demonstrated that plasma ctDNA concentration was significantly associated with both cancer stage and the number of metastatic sites.¹⁷ Jia *et al.* showed that higher ctDNA quantities are correlated with bone metastasis.²⁰ Similarly, this study showed that the detection of plasma ctDNA is significantly correlated with distant metastasis, especially in bone and brain metastasis.

In this study, five patients were *EGFR* mutation positive in plasma but negative in tissue samples. All false-positive plasma samples resulted with L858R mutation and fractional abundance was 0.03, 0.12, 0.13, 0.13 and 0.16, respectively. Although the possibility of experimental artifacts could not be totally excluded, these inconsistencies in the *EGFR* mutation status between tissue and blood samples may be due to the heterogeneity of the tumor.²¹ Oxnard *et al.* revealed the presence of resistance point mutation T790M mutation in 78% of the 18 discordant cases (plasma positive/tissue negative).²² This study also showed that patients who were positive for T790M in plasma had the same treatment outcomes with third-generation TKIs (eg. osimertinib) as patients where the mutation was evident in tissue samples. Therefore, even if there is wild-type in tissue samples, close monitoring of prognosis is necessary if the patient's blood sample is mEGFR positive. In our data, five cases of false-positive cases were reported: two in stage I, one in stage II, and two in stage IV. One patient with stage IV cancer was lost to follow-up five months after diagnosis. Four patients were diagnosed three months before data collection, and are still under follow-up without recurrence or death. Additional follow-up is required to determine whether this difference (wild-type in tissue and mEGFR in blood) is associated with prognosis of lung cancer.

This analysis also reported that 22 patients showed *EGFR*-mutation in tissue but not in the paired plasma sample. Of the false-negative cases, three received EGFR-TKIs and had median PFS of 19 months. There was a longer duration of disease control by EGFR-TKIs in the false-negative group than the overall EGFR-TKI treated group

(19.0 vs. 13.0 months). This is probably due to the low mutation burden, which is likely to result in a low concentration of ctDNA released into the plasma and not detected in it. To determine whether these discordant results between tissue and blood sample affect the prognosis of NSCLC, additional data and analysis are required.

Recent reports suggested that presence of mEGFR ctDNA is associated with longer PFS and OS in advanced NSCLC patients treated with EGFR-TKIs.^{23,24} In this study, poor PFS and a trend toward shorter OS times were observed in lung adenocarcinoma patients with plasma mEGFR ctDNAs. In addition, this analysis also showed that *EGFR* mutations in ctDNA were associated with shorter duration of disease control in advanced lung adenocarcinoma patients treated with EGFR-TKIs. Similar to our results, Mok *et al.* and Lee *et al.* found that EGFR mutations in ctDNA were correlated with poor PFS and OS in NSCLC patients treated with EGFR-TKIs, suggesting that presence of plasma mEGFR ctDNA predicts a poor clinical outcome.^{14,25}

In conclusion, plasma-based *EGFR* mutation analysis using ddPCR is a highly sensitive and specific test, especially in patients with advanced NSCLC. In addition, the detection of ctDNA with mutant *EGFR* is a biomarker for predicting distant metastasis and poor response to EGFR-TKIs.

Acknowledgments

This work was supported by the National Research Foundation of Korea (grant number NRF-2017R1A2B4009017).

Disclosure

The authors declare that they have no competing interests.

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Supporting Information

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

Figure S1 ROC analysis of plasma ddPCR assay of lung adenocarcinoma tissue samples. (a) Overall stage, (b) Early stage (I–IIIA), and (c) Advanced stage (IIIB–IV) for E19d. (d) Overall stage, (e) Early stage (I–IIIA), and (f) Advanced stage (IIIB–IV) for L858R.

Figure S2 Fractional abundance values from plasma ddPCR assay, according to (a) T stage and (b) N stage.