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Plasma epidermal growth factor receptor mutation testing with a chip-based digital PCR system in patients with advanced non-small cell lung cancer

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ABSTRACT

Objectives: Epidermal growth factor receptor (*EGFR*) mutation testing is a companion diagnostic to determine eligibility for treatment with EGFR tyrosine kinase inhibitors (EGFR-TKIs) in non-small cell lung cancer (NSCLC). Recently, plasma-based EGFR testing by digital polymerase chain reaction (dPCR), which enables accurate quantification of target DNA, has shown promise as a minimally invasive diagnostic. Here, we aimed to evaluate the accuracy of a plasma-based *EGFR* mutation test developed using chip-based dPCR-based detection of 3 *EGFR* mutations (exon 19 deletions, L858R in exon 21, and T790M in exon 20).

Materials and methods: Forty-nine patients with NSCLC harboring EGFR-activating mutations were enrolled, and circulating free DNAs (cfDNAs) were extracted from the plasma of 21 and 28 patients before treatment and after progression following EGFR-TKI treatment, respectively.

Results: Using reference genomic DNA containing each mutation, the detection limit of each assay was determined to be 0.1%. The sensitivity and specificity of detecting exon 19 deletions and L858R mutations, calculated by comparing the mutation status in the corresponding tumors, were 70.6% and 93.3%, and 66.7% and 100%, respectively, showing similar results compared with previous studies. T790M was detected in 43% of 28 cfDNAs after progression with EGFR-TKI treatment, but in no cfDNAs before the start of the treatment.

Conclusion: This chip-based dPCR assay can facilitate detection of EGFR mutations in cfDNA as a minimally invasive method in clinical settings.

Abbreviations: ARMS, amplification refractory mutation system; BEAMing, beads, emulsions, amplification and magnetics; cfDNA, circulating free DNA; CI, confidence interval; ddPCR, droplet digital polymerase chain reaction; dPCR, digital polymerase chain reaction; EGFR, epidermal growth factor receptor; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitors; JAK2, Janus kinase 2; NSCLC, non-small cell lung cancer; PFS, progression-free survival; RR, response rate; SD, standard deviation.

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1. Introduction

Epidermal growth factor receptor (*EGFR*) mutation testing is essential for treatment decisions for patients with advanced non-small cell lung cancer (NSCLC). EGFR tyrosine kinase inhibitors (EGFR-TKIs), including gefitinib, erlotinib, and afatinib, are effective against patients with NSCLC harboring EGFR-activating mutations, such as exon 19 deletions or the L858R mutation in exon 21 [1–6]. However, most patients with NSCLC treated with EGFR-TKIs eventually acquire resistance. The T790M mutation in *EGFR* exon 20 causes approximately 50% of acquired resistance to EGFR-TKIs in patients [7,8]. Recently, the third-generation EGFR-TKI osimertinib, which specifically targets EGFR T790M, was approved for use in some countries including the US and Japan, and the importance of serial biopsies for detecting T790M is increasing, as such information determines the appropriateness of osimertinib treatment [9,10]. However, repeated biopsies are sometimes highly invasive and can be difficult to perform without complications [11].

Recently, some studies have reported the efficacy of *EGFR*-activating mutation analysis with circulating free DNA (cfDNA) extracted from the plasma of patients with NSCLC [12–21]. Moreover, *EGFR*-activating mutation analysis with cfDNA was also approved as a companion diagnosis for selecting patients eligible for treatment with gefitinib and osimertinib in the European Union. However, some technical limitations for detecting *EGFR* mutations with cfDNA have been reported. For example, the quantity and quality of circulating tumor-derived DNA varies widely between patients [22]. Moreover, the detectable percentage of the tumor-derived DNA fraction in cfDNA can reach as low as 0.01% [23]. The digital polymerase chain reaction (dPCR), which enables accurate copy-number quantification of target molecules from low-input DNA, is thought to be a promising technology for overcoming the above limitations in mutation testing with cfDNA [24]. The high performance of dPCR is achieved by compartmentalizing a sample at the level of a single DNA molecule by distributing a sample into thousands of separate PCR reactions. Accordingly, the total copy number of targets can be determined by counting the positive and negative partitions. dPCR platforms are classified into 3 types based on the compartmentalization method used, including droplet digital PCR (ddPCR) [20,25–30], BEAMing (beads, emulsions, amplification and magnetics) PCR [19,20,31], and chip-based dPCR [30,32,33]. Plasma-based *EGFR* mutation testing with ddPCR has been evaluated in many institutes. However, chip-based dPCR has not been widely evaluated.

The new chip-based dPCR system, QuantStudio 3D (QS3D) Digital PCR System (Thermo Fisher Scientific, Waltham, MA, USA), was launched. The conventional chip-based BioMark dPCR system (Fluidigm, San Francisco, CA, US) compartmentalizes DNA into 9,180 micropores [32,33], whereas the QS3D Digital PCR system compartmentalizes DNA into 20,000 micropores, which is comparable to the compartmentalization ability of the ddPCR platform, QX100/QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA, US) broadly used in studies for liquid biopsy [25–29]. Moreover, both PCR and detection processes can be consecutively performed within a hermetically sealed reaction chamber in the QS3D Digital PCR system; however, this is not possible with the ddPCR system. This feature of the QS3D Digital PCR system offers the advantage of requiring fewer pipetting process than those needed for ddPCR. Together, these properties contribute to the reduced risk of cross-contamination. The QS3D Digital PCR system runs each chip individually, indicating suitability of the QS3D Digital PCR system for clinical settings in small institutions without vast amounts of samples. In previously studies, this QS3D Digital PCR system has been used to detect DNA mutations in Janus kinase 2 (JAK2) [34] and coagulation factor V genes [35], microRNAs [36], and viral DNA [37], but not *EGFR* mutations.

Therefore, this study aimed to evaluate plasma-based *EGFR* mutation analysis in patients with advanced NSCLC, using this new chip-based dPCR system, QS3D Digital PCR System.

2. Material and methods

2.1. Patients

All patient samples used in this study were collected from our previous prospective study to evaluate plasma *EGFR* mutation testing with the RNase H-dependent PCR and blocking oligo-dependent PCR methods [38]. The present study was designed to evaluate the performance of the QS3D Digital PCR System in plasma-based *EGFR* mutation analysis, using cfDNA samples archived in that previous study [38]. To be eligible for our previous study, patients needed to have been diagnosed with advanced-stage NSCLC or post-operative recurrence and to have an EGFR-activating mutation in their tumors, as confirmed by the Scorpion ARMS (amplification refractory mutation system) method [39]. In our previous study [38], 49 patients were enrolled from October 2013 to March 2014, and tumor staging was evaluated according to the Seventh Edition of TNM in Lung Cancer [40]. Clinical factors at the time of blood sampling were obtained from the patients' medical records. Based on the timing of blood sampling, patients were divided into 2 groups. One group included 21 patients enrolled before the start of EGFR-TKI treatment, and the other group included 28 patients who showed disease progression after EGFR-TKI treatment. Written informed consent was obtained from all patients. This study was conducted in accordance with the provisions of the Declaration of Helsinki and was approved by the Institutional Review Board of Shizuoka Cancer Center (approval number #20-50-27-2-3).

2.2. Chip-based dPCR

Plasma samples and DNA extraction are described in the Supplementary Materials and Methods. dPCR was performed on the QS3D Digital PCR System (Thermo Fisher Scientific), which was composed of a ProFlex PCR system, a QS3D digital PCR chip loader, and a QS3D chip scanner (all from Thermo Fisher Scientific).

To detect the most common deletions in *EGFR* exon 19, a pair of primers and 2 TaqMan probes were designed, based on Yung's report [32] (Supplementary Fig. 1). As a reference probe, a VIC-labeled probe was designed against a region neighboring the exon 19 deletion sites that lacks reported mutations. A wild-type-specific FAM probe was designed against the region where most exon 19 deletions occur. In samples harboring exon 19 deletions, the FAM probe cannot anneal; thus, only VIC fluorescence is detected. The sequences of the amplification primers and TaqMan probes used are given in Supplementary Table 1. To detect the L858R and T790M mutations, predesigned TaqMan probe and primer sets, AHRSRSV (EGFR 6224) and AHRSROS (EGFR 6240), respectively, were purchased from Thermo Fisher Scientific.

2.3. Assessment of the detection limit for each mutation

The detection limit of each assay was defined as the lowest target concentration that could be specifically detected (i.e., with no false-positive detection in the absence of the target) and was determined using 20 ng sample on each of 2 chips containing HDx Reference Standard DNA (Horizon Discovery, Cambridge, UK), which was validated using a ddPCR system of Bio-Rad. The reference standard DNAs used included HD251 ΔE746-A750 (registered in the COSMIC database as the most frequent exon 19 deletion [http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/]), HD254 for L858R, and HD258 for T790M. Each reference mutant DNA contains each mutant sequence at a frequency of 50%. Each reference mutant DNA

Table 1
Patient characteristics.

	All patients (n = 49) (%)	Patients before start of EGFR-TKI treatment (n = 21) (%)	Patients with PD after EGFR-TKI treatment (n = 28) (%)
Age, years			
median	67	74	65
range	42–89	50–89	42–81
Sex			
Male	20 (41)	7 (33)	13 (46)
Female	29 (59)	14 (67)	15 (54)
Smoking history			
Never	28 (57)	16 (76)	12 (43)
Current/Former	21 (43)	5 (24)	16 (57)
Histology			
Adenocarcinoma	49 (100)	21 (100)	28 (100)
EGFR mutation			
Ex19 del	34 (69)	14 (67)	20 (71)
L858R	15 (31)	7 (33)	8 (29)
Stage			
IIIB/IV	41 (84)	18 (86)	23 (82)
Postoperative recurrence	8 (16)	3 (14)	5 (18)

EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; PD, progressive disease; Ex19 deletion, exon 19 deletion mutation; L858R, L858R mutation in exon 21.

was serially diluted with the corresponding wild-type *EGFR* reference DNA (HD709, Horizon Discovery) to 50%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, or 0.01%. We regarded the performance of dPCR as adequate only if the serially diluted DNA copies were detected, as expected. The average copy number detected in the presence of wild-type *EGFR* reference DNA (i.e., negative control) plus 3 standard deviations (SD) was used as the cut-off value for each mutation site.

2.4. Statistical analysis

The sensitivity and specificity of the chip-based dPCR assays in detecting *EGFR* mutations were evaluated by comparing results obtained with the Scorpion ARMS method using the corresponding tumor samples (the reference method). The 95% confidence intervals (CI) for the sensitivity and specificity were computed using the Clopper and Pearson method [41]. Fisher's exact test was used to examine whether the test results correlated with clinicopathological characteristics. Progression-free survival (PFS) was defined as the duration between the initiation of EGFR-TKI therapy and the onset of progressive disease or death from any cause. PFS was calculated according to the Kaplan–Meier method and compared using the log-rank test. Correlation coefficients were calculated using Spearman's rank analysis. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using JMP software, version 12.0.1 for Windows (SAS Institute, Cary, NC, US) and R software, version 3.1.3 (http://www.r-project.org).

3. Results

3.1. Patient characteristics

The patient characteristics are shown in Table 1. Thirty-four patients with NSCLC harbored an exon 19 deletion and 15 patients presented the L858R mutation in their tumor samples, as detected using the Scorpion ARMS method. Twenty-one blood samples were collected from patients who had never been treated with EGFR-TKIs, and 28 blood samples were collected from patients with progressive disease after treatment with EGFR-TKIs. The patients had a median age of 67 years (range 42–89 years), and 29 patients (59%) were female. Twenty-eight patients (57%) were never-smokers, and 21 (43%) had a smoking history. Forty-one patients (84%) had stage IIIB or stage IV disease, and 8 patients (16%) had post-operative recurrence. All lung tumors were classified histologically as adenocarcinoma.

Table 2
Comparison of *EGFR* exon 19 deletion and L858R status in cfDNA and paired tumor samples.

	Tumor tissue				
Plasma cfDNA	+	–	Sensitivity	Specificity	Concordance
Exon 19 del					
+	24	1	70.6%	93.3%	77.6%
–	10	14			
L858R					
+	10	0	66.7%	100.0%	89.8%
–	5	34			

cfDNA, circulating free DNA; Ex19 del, exon 19 deletion mutation; L858R, L858R mutation in exon 21; M+, mutation positive; M-, mutation negative.

3.2. Determination of assay performance

After preparing 7 serial dilutions of genomic DNA harboring an *EGFR* mutant in genomic DNA with wild-type *EGFR* (range: 50%–0.01% mutant *EGFR*), we stably detected the targeted *EGFR* mutations at a frequency as low as 0.1% (Supplementary Fig. 2). Experiments were repeated over 3 separate days. To determine the cut-off value, reference DNA with wild-type *EGFR* was analyzed 8 times using amplification primers and each mutation-specific probe. The average copy numbers for the wild-type reference DNA (plus 3 SDs), which were used as the cut-off values, were 1.6, 2.1, and 0 copies/assay for exon 19 deletions, L858R, and T790M, respectively. Supplementary Fig. 3A shows comparisons between the expected mutation frequency in 20 ng of DNA loaded into 2 separate chips vs. the observed copy numbers of *EGFR* mutations. Mutant alleles were stably detected using these cut-off values, down to a prevalence of 0.1% (Supplementary Fig. 3A). We also evaluated the copy number of *EGFR* mutations in plasma samples using these determined cut-off values. A positive linear relationship between the expected and observed mutation frequencies was observed over a range of 0.1–50% during testing for all mutants examined. Regression analysis showed coefficients of determination (R^2) of 0.9976, 0.9952, and 0.9961 with detection of the exon 19 deletion mutant, L858R, and T790M, respectively (Supplementary Fig. 3B).

3.3. EGFR mutation testing in clinical plasma cfDNA samples

Among 49 patients, the median concentration of cfDNA isolated per ml of plasma across all samples was 35.1 ng/ml (range 12.2–139.5). Twenty-five plasma samples were identified as positive for an exon 19 deletion, based on the cut-off copy number (>1.6), and 10 were identified as positive for L858R (cut-off copy number >2.1), as shown in Fig. 1 and Supplementary Table 2. Only 1 plasma sample was positive for both an exon 19 deletion and L858R, although only the L858R mutation was detected in the corresponding tumor tissue. The sensitivity and specificity of each dPCR assay calculated by comparison with the corresponding tumor samples were 70.6% (24/34; 95% CI, 52.5%–84.9%) and 93.3% (14/15; 95% CI, 68.1%–99.8%) for exon 19 deletion, and 66.7% (10/15; 95% CI, 38.4%–88.2%) and 100% (34/34; 95% CI, 89.7%–100%) for L858R (Table 2). The overall concordance rate between the plasma and tumor tissues was 77.6% (38/49; 95% CI, 63.4%–88.2%) for exon 19 deletion and 89.8% (44/49; 95% CI, 77.8%–96.6%) for L858R.

Twelve samples were identified as positive (cut-off copy number >0) for the T790M mutation (Fig. 1 and Supplementary Table 2). All plasma samples with the T790M mutation were obtained from patients showing progression after EGFR-TKI treatment (43%;

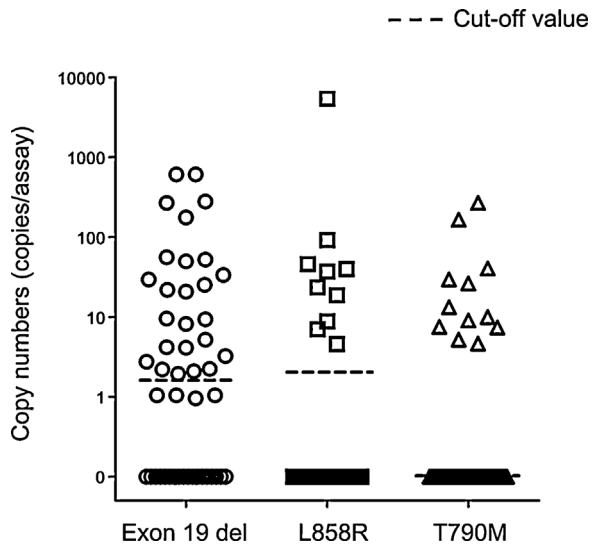

Fig. 1. Distribution of *EGFR* mutant-allele copy numbers per assay in plasma samples. In the exon 19 deletion assay, the copy numbers detected in plasma samples ranged from 1.0 to 610.7 copies/assay, and 25 plasma samples were scored as positive using a cut-off value of 1.6 copies/assay. In the L858R assay, the copy numbers detected in plasma ranged from 4.6 to 5380.4 copies/assay, and 10 samples were scored as positive for L858R using a cut-off value of 2.1 copies/assay. In the T790M assay, the copy numbers measured in plasma samples ranged from 4.6 to 267.2 copies/assay, and 12 plasma samples were scored as positive using a cut-off value of 0 copies/assay. The dashed line shows the average copy numbers for negative samples plus 3 × SD as the cut-off value. Exon 19 del, exon 19 deletion; L858R, L858R mutation in exon 21; T790M, T790M mutation in exon 20.

12/28), with the T790M mutation not being observed in patient samples obtained before EGFR-TKI treatment.

In 28 patients with progressive disease after treatment with EGFR-TKIs, 11 out of 12 patients with NSCLC and detectable EGFR T790M in the plasma underwent re-biopsy, 5 of which also presented the T790M mutation in their re-biopsy samples (Supplementary Tables 2 and 3). In addition, 9 out of 16 patients with NSCLC lacking detectable T790M in the plasma underwent re-biopsy, and 2 patients presented T790M in their re-biopsy samples (Supplementary Tables 2 and 3).

Among the 25 patients with an exon 19 deletion in the plasma, the percent mutation rate in the plasma ranged from 0.3% to 45.4% (median 2.3%). Among the 10 patients with detectable L858R in the plasma, the percent mutation rate in the plasma ranged from 0.5% to 70.8% (median 2.4%), including 1 patient who had both an exon 19 deletion and the L858R mutation. Among the 12 patients with detectable T790M in the plasma, the percent mutation rate in the plasma ranged from 0.5% to 11.6% (median 1.4%). No statistically significant relationships between the amount of input cfDNA and the copy numbers of each *EGFR* mutation were observed (Supplementary Fig. 4).

3.4. *Association between* EGFR *mutation detection and extra-thoracic metastatic disease*

Among all patients, the relationship between the detection of *EGFR* mutations in cfDNA and the existence of extra-thoracic metastases was evaluated. *EGFR*-activating mutations tended to be more frequently detected in the plasma of patients with extra-thoracic metastatic disease (M1b) than in patients with disease confined to the thoracic cavity (M1a/M0; p = 0.053; Table 3). No difference in the detection of the *EGFR* T790M mutation in the plasma was observed between patients with M1a/M0 and M1b disease classifications (p = 0.29).

Table 3
Relationship between EGFR-activating mutations and NSCLC classification.

		Disease classification		
Plasma cfDNA		M1a/M0	M1b	Total
EGFR-activating mutations	+	8	26	34
	–	8	7	15
	Total	16	33	49
				P = 0.053

cfDNA, circulating free DNA.

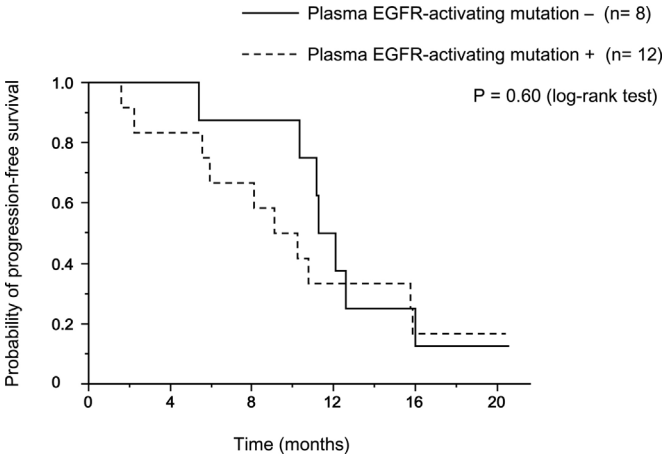

Fig. 2. Progression-free survival (PFS) of 20 patients with NSCLC who enrolled in this study before the start of EGFR-TKI treatment. The median PFS was 9.7 months in patients with detectable EGFR-activating mutations in the plasma (dashed line), and 11.7 months in patients without detectable EGFR-activating mutations in the plasma (solid line; p = 0.60).

3.5. *Plasma* EGFR *mutation status at pre-treatment vs. clinical outcomes*

Among the 21 patients who enrolled before the start of EGFR-TKI treatment, 20 patients were treated with EGFR-TKIs after plasma sampling. No statistically significant difference was observed in the response rate (RR) and PFS following EGFR-TKI treatment between patients with detectable (n = 12) and undetectable (n = 8) *EGFR*-activating mutations in the plasma (RR: 87.5 vs. 66.7%, p = 0.60; median PFS: 11.7 vs. 9.7 months, p = 0.60; Fig. 2).

4. Discussion

To our knowledge, this is the first study to evaluate the performance of detection assays for exon 19 deletion, L858R, and T790M in plasma samples using the QS3D Digital PCR System. *EGFR* mutations were stably detected at a prevalence as low as 0.1%, indicating consistency with data from a previous study for detection of JAK2 V617F mutation in myeloproliferative neoplasms with the QS3D Digital PCR system [34]. The detection limits of ddPCR, using the QX100/QX200 Droplet Digital PCR System were estimated as 0.01–0.04% [25–29]. Similarly, the detection limit of BEAMing PCR was estimated as 0.01% [31], and that of chip-based dPCR using the BioMark system was 0.03–0.1% [32,33] (Table 4). As shown in Table 4, we observed similar sensitivity and specificity compared to the other dPCR assays. These results indicated that the lower detection limit did not correlate with the sensitivity and specificity in detecting *EGFR* mutations in plasma. This discrepancy might have resulted from the use of different types of DNA, such as plasmid and human genomic DNA, when determining the lower detection limit. Moreover, it is possible that differences in the design of the primers and probes, as well as the cut-off value selected, also caused the differences in the results.

Table 4
Performance comparison with other PCR methods for evaluating *EGFR* mutations in plasma.

Method	Detection limit (%)	Sensitivity (%)	Specificity (%)
Allele-specific PCR (Cobas) [18–21]	0.1	60.7–84.3	96.4–97.0
BEAMing PCR [19,20,30]	0.01	72.7–87.0	96.8–100
Droplet dPCR [25–29]	0.01–0.04	66.7–84.3	95.8–100
Chip-based dPCR [31,32]	0.03–0.1	78.9	100
This study	0.1	66.7–70.6	93.3–100

dPCR, digital PCR.

EGFR-activating mutations tended to be more frequently detected in the plasma of patients with extra-thoracic metastatic disease (M1b) than in patients with limited thoracic disease (M1a/M0; p = 0.053; Table 3). These results are consistent with those of previous studies and suggest that the tumor burden or metastatic status may influence the detection of *EGFR* mutations in the plasma [15,19,20,42]. Although no statistically significant differences were observed in the RR and PFS following EGFR-TKI treatment between patients with or without detectable *EGFR*-activating mutations in their plasma, patients with *EGFR*-activating mutations in the plasma appeared to have inferior PFS (Fig. 2). These results might have been influenced by the metastatic status.

While a low frequency of de novo T790M mutations in tumor samples was reported in one study [43], another study showed the existence of the T790M mutation in approximately half of the tumor samples tested after progression with EGFR-TKI treatment [8]. In the present study, T790M mutations were only detected in plasma samples of patients showing disease progression after EGFR-TKI treatment, and the frequency of T790M detection in plasma samples was 43%, similar to data from previous dPCR studies showing that 28.6%–66.7% of patients with NSCLC had detectable T790M in the plasma [19,20,26,28,29,33]. Two previous reports showed that the clinical response rates for third-generation EGFR-TKIs in patients positive for the T790M mutation in the plasma were nearly identical to that in patients with positive tissue samples [19,20]. These results suggest that plasma T790M can serve as a biomarker for the use of third-generation EGFR-TKIs; thus, it will become more important to test for the presence of plasma T790M in future clinical settings.

This study has some limitations. Firstly, we could not perform the comparison assay with the same cfDNA samples between the QS3D Digital PCR system and other dPCR platforms. Instead, in this study, we performed the assessment of assay performance of this QS3D Digital PCR assay with commercially available certified reference DNA materials whose mutant allele copy numbers were validated using a ddPCR system of Bio-Rad (Supplementary Figs. 2 and 3). Secondly, because enough matching tumor samples were not available to evaluate the QS3D Digital PCR assay, the detection of *EGFR*- mutations by dPCR was performed only with plasma samples and not with tissue samples. The sensitivity and specificity of the assays were calculated according to the *EGFR* mutation status of tumor samples, as first determined using the Scorpion ARMS method. Thirdly, differences occurred in the timing between blood sampling and tumor tissue sampling for some patients. The prevalence of EGFR-activating mutations in the plasma can be influenced by treatment with EGFR-TKIs or cytotoxic chemotherapies [25]. The time course may also have an influence on the level of *EGFR*-activating mutations detected in the plasma. Accordingly, we could not evaluate the concordance of T790M mutations between plasma and tissue samples. The level of the T790M mutation present in plasma may change following EGFR-TKI treatment [25]. In this study, T790M mutations were only detected in the plasma of patients showing progression after EGFR-TKI treatment; thus, the T790M mutation status in the corresponding tumor samples at the time of blood sampling is unclear. However, this is also generally a limitation in clinical settings. These limitations might also be associated with the causes owing to which the performance of the QS3D Digital PCR system was similar to that of the conventional chip-based BioMark dPCR system (Fluidigm), which has fewer micropores compared to the QS3D Digital PCR system [32], although a previous study reported that the number of partitions impacts the measurement precision and dynamic range [44]. To overcome the above-mentioned limitations, prospective studies with time-matched blood and tissue samples obtained from more patients with lung adenocarcinoma are necessary for further evaluation of the performance of the EGFR mutation-detection system developed in this study.

5. Conclusion

We evaluated dPCR assays for detecting *EGFR* mutations in plasma samples of patients with NSCLC using the QS3D Digital PCR System. This system showed similar performance to other dPCR assays. Our results indicated that *EGFR* mutation testing with cfDNA using chip-based dPCR can be useful as a minimally invasive monitoring method in clinical settings.

Conflict of interest

The authors have the following conflicts of interest to declare: H. K., Y. H., M. Y., and T. T. received research grants from AstraZeneca. K.K. H. K., H. M., Y. K., M. Y., and T. T. received speaking fees from AstraZeneca. K.K. H. K., Y. K., and M. Y. received research grants from Boehringer Ingelheim, Japan. H. K., H. M., Y. K., M. Y., and T. T. received speaking fees from Boehringer Ingelheim, Japan. Y. H., Y. K., M. Y., and T. T. received research grants from Chugai Pharmaceutical Co., Ltd. H. K., A. O., S. O., H. M., M. Y., and T. T. received speaking fees from Chugai Pharmaceutical Co., Ltd. H. K., A. O., and S. O. received speaking fees from Taiho Pharmaceutical Co., Ltd. T. T. received research grants from Eli Lilly Japan K.K. H. K. and A. O. received speaking fees from Eli Lilly Japan K.K. T. T. received research grants from Pfizer Japan, Inc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2017.02.001.

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