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ORIGINAL ARTICLE

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Exploration of resistance mechanisms for epidermal growth factor receptor-tyrosine kinase inhibitors based on plasma analysis by digital polymerase chain reaction and next-generation sequencing

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Liquid biopsy offers a potential alternative to tissue biopsy for detection of genetic alterations in cancer, and it has been introduced into clinical practice to detect the tyrosine kinase inhibitor (TKI) resistance-conferring T790M mutation of epidermal growth factor receptor (*EGFR*) in patients with non-small-cell lung cancer (NSCLC). We prospectively collected tumor and plasma samples from 25 NSCLC patients who harbored activating mutations of *EGFR* and experienced failure of treatment with afatinib. The samples were analyzed by digital PCR (dPCR) and next-generation sequencing (NGS). T790M was detected in plasma with a respective sensitivity and specificity of 83.3% and 70.0% by dPCR and 50.0% and 70.0% by NGS relative to analysis of corresponding tumor samples. Quantitation of T790M based on the ratio

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of the number of T790M alleles to that of activating mutation alleles (T/A ratio) improved the specificity of plasma analysis to 100% for both dPCR and NGS without a reduction in sensitivity. Although several afatinib resistance mechanisms other than T790M—including copy number gain of *NRAS* or *MET*—were identified in tumor samples, the corresponding genetic alterations were not detected in plasma. *TP53* mutations were frequently identified in plasma and tumor samples, with most such mutations also having been detected before afatinib treatment. The presence of de novo *TP53* mutations was associated with reduced progression-free survival. Quantitation of T790M in plasma is thus a clinically relevant approach to determine the T790M status of tumors. In addition, genetic alterations coexisting with *EGFR* mutations can affect the efficacy of EGFR-TKI treatment.

KEYWORDS
afatinib, circulating tumor DNA, digital PCR, next-generation sequencing, resistance mechanism

1 | INTRODUCTION

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) show marked efficacy for treatment of non-small-cell lung cancer (NSCLC) positive for activating mutations of *EGFR*.[1-7] Isolation of circulating cell-free DNA (cfDNA) from blood samples as a liquid biopsy can be carried out in cancer patients in a noninvasive and repetitive way, with analysis of the cfDNA having the potential to provide insight into the extent of intratumoral heterogeneity.[8,9] Such an approach has been introduced into clinical practice to detect activating mutations or the TKI resistance-conferring T790M mutation of *EGFR* in patients with NSCLC.[10-16] Monitoring of cfDNA by digital PCR (dPCR) has been shown to be informative for prediction of EGFR-TKI efficacy, whereas that by next-generation sequencing (NGS) has the potential to identify mechanisms of treatment resistance.[16] Liquid biopsy thus offers a promising alternative to tissue biopsy both for the detection of genetic alterations that can inform the selection of corresponding targeted drugs and for exploration of mechanisms of resistance to such drugs.

Third-generation EGFR-TKI osimertinib prolonged progression-free survival (PFS) compared with platinum chemotherapy plus pemetrexed in patients with NSCLC positive for activating mutations of *EGFR* who acquired the T790M mutation and whose disease progressed during previous EGFR-TKI therapy.[17] Detection of T790M at the time of progression is therefore essential for determination of the optimal subsequent treatment for this patient population. Given that tumor biopsy is invasive and not always feasible, liquid biopsy could be an important alternative for such analysis. Analysis by NGS has the potential to detect several genetic alterations such as *TP53* mutations that exist together with *EGFR* mutations, but little is known of how such coexisting genetic alterations affect clinical outcome.[18,19]

We previously showed that monitoring of cfDNA by dPCR is informative for prediction of the efficacy of the second-generation EGFR-TKI afatinib in NSCLC patients positive for *EGFR* activating mutations and that allele frequency for somatic mutations in cfDNA determined by NGS changed concordantly during afatinib treatment with the number of *EGFR* mutant alleles determined by dPCR.[16] In the present study, both tumor and plasma samples were prospectively collected from these patients at the time of treatment failure and were examined for genetic alterations with both dPCR and NGS in order to investigate mechanisms of resistance to afatinib treatment.

2 | MATERIALS AND METHODS

2.1 | Study design and participants

Between 20 May and 25 November 2014, a total of 35 patients who had not previously been treated with EGFR-TKI were enrolled in the present study from 10 institutions across Japan. The patients had histologically or cytologically confirmed adenocarcinoma of the lung at stage IIIB or IV or postoperative recurrence, and they were positive for a common activating mutation of *EGFR* (an exon 19 deletion [Ex19del] or the L858R point mutation). They received a single daily dose of afatinib at a starting dose of 40 mg until development of progressive disease (PD) or intolerable adverse events, or until withdrawal of consent. Detailed information regarding study design as well as the baseline demographics and clinical characteristics of the patients has been presented previously.[16] All patients provided written informed consent, and the study was carried out in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of each institution.

2.2 | Sample collection

Tumor and plasma samples were collected before afatinib treatment and after treatment failure with disease progression (systemic PD). Blood samples (14 mL) were collected in tubes containing EDTA

(disodium salt) and were centrifuged at 1400 *g* for 10 minutes at room temperature within 1 hour of collection. The supernatant was stored at −80°C until analysis. Detailed protocols for isolation of DNA from tumor samples and from blood samples (cfDNA) are provided in Doc. S1.

2.3 | Scorpion amplification-refractory mutation system

We carried out allele-specific PCR analysis by Scorpion ARMS (amplification-refractory mutation system) with a Therascreen EGFR RGQ PCR Kit (QIAGEN K.K., Tokyo, Japan) as a conventional method to detect activating and T790M mutations of *EGFR* in tumor samples. Details are provided in Doc. S1.

2.4 | Nanofluidic dPCR analysis

The principles and details of the nanofluidic dPCR system (BioMark HD System; Fluidigm, South San Francisco, CA, USA) as carried out with the Fluidigm digital chip have been described previously.[16,20] ARMS was carried out in each reaction chamber targeting either the control region (exon 2), activating mutations (Ex19del or L858R), or T790M of *EGFR* in both tumor and plasma samples. We carried out duplicate assays (two panels) for detection of each target allele. Tumor samples collected from 15 NSCLC patients without *EGFR* activating mutations were analyzed to determine the cutoff for T790M positivity in tumor samples (Table S1). The number of positive signals observed in the T790M reaction panels increased with the estimated number of control alleles applied (Figure S1). A tumor sample was thus considered positive for T790M if the ratio of the number of positive signals in the T790M reaction to the number of control alleles was ≥0.54 (mean + 3 SD; Table S1). The concentration of cfDNA in plasma samples collected either from five patients with NSCLC negative for *EGFR* activating mutations or from five healthy donors was much lower than that of tumor DNA, with the result that essentially no signal for T790M was detected with the plasma samples (Table S2). We therefore set a cutoff for T790M positivity in plasma samples different from that in tumor samples. On the basis of this analysis, plasma samples were thus considered positive for T790M if the ratio of the number of positive signals in the T790M reaction to the number of control alleles was ≥0.17 (mean + 3 SD).

2.5 | Next-generation sequencing analysis

Tumor and cfDNA samples were analyzed with NGS panels for mutation detection. For library preparation, tumor DNA (10 ng) and cfDNA (maximum of 3000 copies) were subjected to multiplex PCR amplification with the use of an Ion AmpliSeq Library Kit 2.0 (Life Technologies, Carlsbad, CA, USA) and Ion AmpliSeq Colon and Lung Cancer Panel v2 (Life Technologies), the latter of which targets 22 cancer-associated genes, with 92 amplicons covering 1205 hotspot mutations. Details are provided in Doc. S1.

2.6 | Statistical analysis

Details are provided in Doc. S1.

3 | RESULTS

3.1 | Patient population and clinical outcomes

We have previously indicated marked antitumor activity and tolerable toxicity of afatinib treatment in a prospective study.[16] We updated the data at a median follow-up time of 15.8 months (range, 0.9-41.7 months) as a result of an increase in events related to PFS. Of the 35 patients enrolled in the study, 27 individuals (77.1%; 95% CI, 63.2%-91.1%) showed a partial response and 31 (88.6%; 95% CI, 78.0%-99.1%) achieved disease control. Thirty-four (97.1%) patients discontinued treatment as a result of systemic PD (n = 28, 80.0%), adverse events (n = 5, 14.3%), or treatment refusal (n = 1, 2.9%), whereas one (2.9%) patient was still receiving treatment with afatinib. Median PFS was 14.3 months (95% CI, 10.3-19.8 months; Figure S2A), with PFS for patients with an Ex19del mutation of *EGFR* being slightly longer than that for those with the L858R mutation (15.1 vs 10.8 months, $P = 0.88$; Figure S2B). Median time to systemic PD was 16.3 months (95% CI, 10.8-20.4 months; Figure S2C), with the value for patients with an Ex19del mutation being slightly greater than that for those with the L858R mutation (17.9 vs 12.8 months, $P = 0.87$; Figure S2D). All 35 patients received afatinib at a starting dose of 40 mg/d. Twenty-six (74.3%) patients required a dose reduction, with 13 (37.1%) requiring a dose reduction to 20 mg/d (data not shown). All adverse events leading to a dose reduction were due to nonhematological toxicity, with the most common such events including diarrhea, rash or acne, stomatitis, and nail effect (data not shown).

3.2 | Evaluation of activating mutations and T790M of *EGFR* in tumor and plasma samples at development of systemic PD

For the 28 patients who experienced systemic PD, tumor or plasma samples were collected at the time of PD development from 25 individuals (17 with an Ex19del and 8 with L858R), with tumor, plasma, or both samples being available for 18, 23, and 16 individuals, respectively (Table 1). Among the 18 patients for whom tumor samples were available, *EGFR* activating mutations were identified in 16 (88.9%) and 17 (94.4%) individuals by dPCR and NGS, respectively (Table S3). In the case of the two patients for whom *EGFR* activating mutations were not detected in tumor samples by dPCR, such a mutation was also not detected by NGS in one patient (patient #12). Given that an *EGFR* activating mutation was detected in the tumor sample obtained from this patient before afatinib treatment, the mutation may have disappeared during treatment or the tumor sampling sites may have differed between before and after failure of afatinib treatment (intratumoral heterogeneity). An activating mutation was not detected by dPCR in patient #7 because the mutation

TABLE 1 Concordance of T790M positivity between tumor and plasma samples as evaluated by dPCR, NGS, and ARMS (n = 25)

Patient	Activating mutation identified before afatinib treatment	Tumor samples (n = 18)			Plasma samples (n = 23)	
		dPCR	NGS	ARMS	dPCR	NGS
1	p.E746_A750 del	Yes	Yes	Yes	No	No
2	p.E746_A750 del	Yes	Yes	No	Yes	Yes
3	p.E746_A750 del	Yes	Yes	Yes	Yes	Yes
4	L858R	Yes	Yes	Yes	Yes	No
5	L858R	Yes	Yes	Yes	Yes	Yes
6	p.E746_A750 del	Yes	Yes	Yes	Yes	Yes
7	p.S752_I759 del	Yes	Yes	Yes	Yes	No
8	p.E746_A750 del	No	No	No	No	No
9	p.E746_A750 del	No	No	No	No	No
10	p.E746_A750 del	No	No	No	No	No
11	p.E746_A750 del	No	No	No	Yes	Yes
12	p.E746_S752>V	No	No	No	No	No
13	p.E746_A750 del	No	Yes	No	No	No
14	p.E746_A750 del	No	No	No	No	No
15	L858R	No	No	No	Yes	Yes
16	L858R	No	No	No	No	No
17	L858R	Yes	Yes	Yes	NA	NA
18	p.L747_P753>S	Yes	Yes	Yes	NA	NA
19	L858R	NA	NA	NA	No	No
20	p.E746_A750 del	NA	NA	NA	Yes	Yes
21	p.E746_A750 del	NA	NA	NA	Yes	No
22	p.E746_A750 del	NA	NA	NA	Yes	Yes
23	p.E746_A750 del	NA	NA	NA	Yes	Yes
24	L858R	NA	NA	NA	No	No
25	L858R	NA	NA	NA	Yes	Yes
T790M positivity		9 Yes (50.0%)	10 Yes (55.6%)	8 Yes (44.4%)	13 Yes (56.5%)	10 Yes (43.5%)

Discordant results among dPCR, NGS, and ARMS for tumor and plasma analysis are shaded in gray.
ARMS, amplification-refractory mutation system; dPCR, digital PCR; NA, not available; NGS, next-generation sequencing.

type (Ex19del, p.S752_I759 del) is not recognizable by dPCR. The T790M mutation of *EGFR* was detected in nine (50.0%), 10 (55.6%), and eight (44.4%) of the 18 tumor samples obtained at the time of systemic PD development by dPCR, NGS, and ARMS, respectively (Table 1, Table S3).

Among the 23 plasma samples obtained at the time of systemic PD development, *EGFR* activating mutations were identified in 16 (69.6%) and 14 (60.9%) samples by dPCR and NGS, respectively (Table S3). The T790M mutation was detected in 13 (56.5%) and 10 (43.5%) of these 23 samples by dPCR and NGS, respectively (Table 1, Table S3). Discordant results among dPCR, NGS, and ARMS for detection of T790M in tumor or plasma samples were obtained for eight (32%) of 25 patients (Table 1). In the case of the 16 patients for whom tumor and plasma samples were both available, sensitivity and specificity for detection of T790M in plasma (relative to that in tumor samples with the conventional method of ARMS) were 83.3% and 70.0%, respectively, for dPCR and 50.0% and 70.0%, respectively, for NGS (Figure 1A).

3.3 | Assessment of the T/A ratio for quantitative evaluation of T790M status in tumor and plasma samples at the time of systemic PD development

Given that a high ratio of the number of T790M alleles to that of activating mutation alleles (T/A ratio) in tumor or plasma samples has been associated with resistance to first-generation EGFR-TKI and with a high efficacy of third-generation EGFR-TKI,[13,21,22] we calculated the T/A ratio for both dPCR and NGS and thereby quantitatively evaluated the T790M status of each sample (Tables 2, 3, S4, and S5). We determined that cutoffs for the T/A ratio of 5.0% in dPCR and of 10.0% in NGS allowed positivity for T790M evaluated by these techniques to fully match in both tumor (Figure S3) and

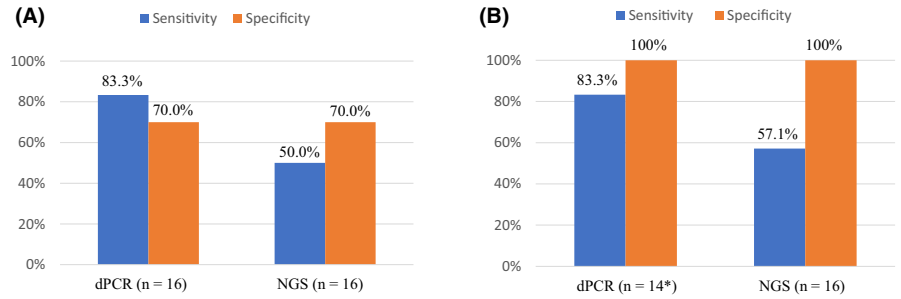

FIGURE 1 Sensitivity and specificity of digital PCR (dPCR) and next-generation sequencing (NGS) for the detection of T790M in plasma samples compared with analysis of tumor samples. Positivity for T790M was determined without the use of the T/A ratio (A) or on the basis of the T/A ratio for plasma and tumor analysis (B). Asterisk indicates that two patients were excluded from the analysis either because the activating mutation (p.S752_I759 del) is not recognizable by dPCR (patient #7) or because the activating mutation seemed to disappear during afatinib treatment (patient #12). T/A ratio, number of T790M alleles to that of activating mutation alleles

plasma (Figure S4) samples. Application of these cutoffs for the T/A ratio improved the specificity of plasma analysis for the detection of T790M (compared with tumor analysis) to 100% for both dPCR and NGS, without a reduction in the sensitivity of either approach (Figure 1B). Such quantitative evaluation of T790M on the basis of the T/A ratio also reduced the number of discordant results among dPCR, NGS, and ARMS for the detection of T790M in tumor or plasma samples to three (12%) out of 25 patients (Table 4). For two (patients #1 and #4) of the three patients with discordant results, T790M was determined to be positive in tumor samples but not in plasma samples, possibly as a result of a low frequency of T790M alleles in the plasma samples. Given the consistent T790M positive results obtained for their tumor samples by dPCR, NGS, and ARMS, these two patients were considered to be T790M positive. For the remaining patient (patient #2) with discordant results, T790M was negative in the tumor sample by ARMS, with a ΔC_t value of 7.49, which is close to the cutoff value of 7.40. We therefore considered this patient to be positive for T790M. On the basis of the various results, 13 (52.0%) of the 25 patients were deemed positive for T790M (Table 4).

3.4 | Next-generation sequencing analysis of somatic alterations other than T790M of *EGFR*

Among the 18 tumor samples obtained at the time of systemic PD development, the T790M mutation of *EGFR* alone (n = 7), copy number gain (CNG) of *NRAS* (n = 1), CNG of *MET* (n = 1), CNG of *EGFR* plus T790M (n = 1), and CNG and E545K of *PIK3CA* plus T790M of *EGFR* (n = 1) were identified by NGS as putative mechanisms of resistance to afatinib (Table 3). CNG of *MET* was also detected in the corresponding tumor sample obtained before treatment, with this patient experiencing systemic PD immediately after afatinib initiation. Putative resistance mechanisms other than T790M were not identified by NGS in plasma samples obtained at the time of systemic PD. Mechanisms of resistance to afatinib including the data for plasma analysis of T790M are summarized in Table 4 and Figure 2. Mutations of *TP53* were identified in tumor or plasma samples obtained at systemic PD from 10 (40%) of the 25 patients with such samples available (Table 3).

3.5 | Survival analysis according to *TP53* mutation and *EGFR* T790M status

We previously identified a total of 45 mutations in tumor samples collected before afatinib treatment from 32 patients with such samples available,[16] with *TP53* mutations being found in 11 (34.4%) of these patients (Table S6). Median PFS was significantly shorter in patients whose tumors harbored a *TP53* mutation before afatinib treatment than in those whose tumors did not (10.3 vs 15.1 months, $P = 0.01$; Figure 3A). Among the 18 patients with tumor samples obtained at the time of systemic PD development, median PFS was slightly longer in those with than in those without T790M as based on the T/A ratio at this time (15.1 vs 10.9 months, $P = 0.25$; Figure 3B). Although the difference also did not achieve statistical significance, median time to systemic PD was substantially longer in patients with T790M as based on the T/A ratio than in those without it (17.9 vs 10.9 months, $P = 0.18$; Figure 3C).

4 | DISCUSSION

In the present study, both tumor and plasma samples were prospectively collected from patients who experienced failure of afatinib treatment and were analyzed by dPCR and NGS. Without application of the T/A ratio, the T790M mutation of *EGFR* was positive in nine (50.0%), 10 (55.6%), and eight (44.4%) of 18 tumor samples obtained at the time of systemic PD development as evaluated by dPCR, NGS, and ARMS, respectively, and it was positive in 13 (56.5%) and 10 (43.5%) of 23 plasma samples by dPCR and NGS, respectively (Table 1). With evaluation of tumor samples by the allele-specific PCR method (ARMS) as reference, the sensitivity and specificity of plasma analysis for T790M were 83.3% and 70.0%, respectively, for dPCR and 50.0% and 70.0%, respectively, for NGS (Figure 1A), values consistent with those reported in previous studies.[13,14]

TABLE 2 Quantitative evaluation of T790M with the T/A ratio in tumor and plasma samples by dPCR (n = 25)

		Tumor samples (n = 18)			Plasma samples (n = 23)		
Patient	Activating mutation identified before afatinib treatment	Activating mutation/control (%)	T790M/control (%)	T/A (%)	Activating mutation/control (%)	T790M/control (%)	T/A (%)
1	p.E746_A750 del	63.36	10.96	**17.30**	1.06	0	0
2	p.E746_A750 del	1.58	0.76	**48.25**	0.39	0.47	**120.00**
3	p.E746_A750 del	120.83	25.00	**20.69**	7.89	0.66	**8.33**
4	L858R	11.04	2.69	**24.32**	0.73	0.37	**50.00**
5	L858R	57.19	14.15	**24.73**	1.83	1.37	**75.00**
6	p.E746_A750 del	28.10	14.05	**50.00**	2.16	0.72	**33.33**
7	p.S752_I759 del	0	10.42	NE[a]	0	0.18	NE[a]
8	p.E746_A750 del	19.51	0	0	0	0	0
9	p.E746_A750 del	52.93	0	0	0	0	0
10	p.E746_A750 del	3.28	0.05	1.52	0	0	0
11	p.E746_A750 del	76.03	0	0	31.77	0.52	1.64
12	p.E746_S752>V	0	0.10	NE[b]	0	0	0
13	p.E746_A750 del	21.69	0.22	1.03	0.43	0	0
14	p.E746_A750 del	48.42	0.53	1.09	0.31	0	0
15	L858R	35.65	0.06	0.16	7.62	0.24	3.16
16	L858R	37.22	0	0	10.20	0	0
17	L858R	82.28	47.58	**57.83**	NA	NA	NA
18	p.L747_P753>S	44.44	6.67	**15.00**	NA	NA	NA
19	L858R	NA	NA	NA	0	0	0
20	p.E746_A750 del	NA	NA	NA	1.75	0.87	**50.00**
21	p.E746_A750 del	NA	NA	NA	1.38	2.07	**150.00**
22	p.E746_A750 del	NA	NA	NA	9.88	5.76	**58.33**
23	p.E746_A750 del	NA	NA	NA	137.72	0.34	0.25
24	L858R	NA	NA	NA	0	0	0
25	L858R	NA	NA	NA	5.48	1.71	**31.25**
T790M positivity				8 Yes (50.0%)[c]			9 Yes (40.9%)[d]

T/A ratios of >5.0% are shown in bold. The T/A ratio was set to zero if both activating mutations and T790M were not detected.
[a]The T/A ratio was not evaluable (NE) in this patient because the activating mutation (p.S752_I759 del) is not recognizable by dPCR.
[b]The T/A ratio was not evaluable in this patient (shaded in gray) because the activating mutation seemed to disappear during afatinib treatment.
[c]Patients #7 and #12 were excluded because of NE status.
[d]Patient #7 was excluded because of NE status.
dPCR, digital PCR; NA, not available; T/A ratio, number of T790M alleles to that of activating mutation alleles.

However, among the 16 plasma samples in which *EGFR* activating mutations were identified at the time of systemic PD development by dPCR, the detection rate for T790M by dPCR was 75.0% (12 of 16; Table S3). Among the 14 plasma samples in which *EGFR* activating mutations were detected at the time of systemic PD development by NGS, the detection rate for T790M by NGS was 71.4% (10 of 14; Table S3). These detection rates for T790M are much higher than those previously reported for tumor samples collected from patients who experienced resistance to first- or second-generation EGFR-TKI.[23-26] Although such a high frequency of T790M might be the result of selection bias due to the small number of samples, we considered the possibility that highly sensitive methods such as dPCR and NGS might detect a small population of T790M alleles in cfDNA that is not responsible for resistance to EGFR-TKI and give rise to the low specificity of plasma analysis for the detection of T790M compared with tumor analysis. Such highly sensitive methods have been found to detect a small proportion of T790M alleles even in specimens obtained from NSCLC patients before treatment

TABLE 3 Quantitative evaluation of T790M with the T/A ratio and additional somatic alterations detected in tumor and plasma samples by NGS (n = 25)

		Tumor samples (n = 18)				Plasma samples (n = 23)			
Patient	Activating mutation identified before afatinib treatment	Allele frequency for activating mutation (%)	Allele frequency for T790M (%)	T/A (%)	Additional somatic alterations	Allele frequency for activating mutation (%)	Allele frequency for T790M (%)	T/A (%)	Additional somatic alterations
1	p.E746_A750 del	52.56	39.56	**75.27**	*CTNNB1*: p.S37Y	0.16	0	0	
2	p.E746_A750 del	1.52	0.94	**62.30**		1.37	0.24	**17.70**	
3	p.E746_A750 del	69.67	16.84	**24.17**		4.08	0.99	**24.24**	
4	L858R	56.36	11.48	**20.37**	*PIK3CA*: p.E545K *PIK3CA* CNG *TP53*: p.Y236*	0	0	0	*TP53*: p.Y236*
5	L858R	73.50	20.74	**28.22**		2.99	0.84	**28.21**	
6	p.E746_A750 del	45.70	18.13	**39.67**		0.54	0.23	**42.77**	
7	p.S752_I759 del	41.54	18.94	**45.60**		1.17	0	0	
8	p.E746_A750 del	22.35	0	0	*TP53*: p.M246L	0	0	0	
9	p.E746_A750 del	63.83	0	0		0	0	0	
10	p.E746_A750 del	10.80	0	0		0	0	0	
11	p.E746_A750 del	71.97	0	0		27.71	0.39	1.39	*TP53*: p.R248G
12	p.E746_S752>V	0	0	0	*TP53*: p.R248W	0	0	0	*TP53* p.R248W
13	p.E746_A750 del	55.04	3.04	5.53	*FGFR3*: p.S378I *NOTCH1*: p.1581R *NRAS* CNG	0	0	0	
14	p.E746_A750 del	44.71	0	0		0.14	0	0	
15	L858R	41.33	0	0	*MET* CNG *TP53*: p.C277F	5.21	0.42	8.12	*TP53*: p.C277F
16	L858R	65.80	0	0		5.73	0	0	
17	L858R	79.40	54.48	**68.61**	*EGFR* CNG *TP53*: p.R273L	NA	NA	NA	
18	p.L747_P753>S	55.52	33.17	**59.74**	*TP53*: p.V274F	NA	NA	NA	
19	L858R	NA	NA	NA		0	0	0	
20	p.E746_A750 del	NA	NA	NA		1.96	1.52	**77.98**	

(Continues)

TABLE 3 (Continued)

Patient	Activating mutation identified before afatinib treatment	Tumor samples (n = 18)				Plasma samples (n = 23)			
		Allele frequency for activating mutation (%)	Allele frequency for T790M (%)	T/A (%)	Additional somatic alterations	Allele frequency for activating mutation (%)	Allele frequency for T790M (%)	T/A (%)	Additional somatic alterations
21	p.E746_A750 del	NA	NA	NA		3.23	0.39	**12.15**	*TP53*: p.D281E
22	p.E746_A750 del	NA	NA	NA		6.96	4.93	**70.86**	*TP53*: p.C242F
23	p.E746_A750 del	NA	NA	NA		50.15	0.32	0.65	*TP53*: p.S241Y
24	L858R	NA	NA	NA		0	0	0	
25	L858R	NA	NA	NA		7.05	2.92	**41.5**	
T790M positivity				9 Yes (50.0%)				8 Yes (34.8%)	

T/A ratios >10.0% are shown in bold. The T/A ratio was set to zero if both activating mutations and T790M were not detected. The *EGFR* activating mutation detected in a tumor sample obtained before afatinib treatment seemed to disappear in patient #12 (shaded in gray). Somatic alterations that had been identified before afatinib treatment are shown in red.
CNG, copy number gain; EGFR, epidermal growth factor receptor; NA, not available; NGS, next-generation sequencing; T/A ratio, number of T790M alleles to that of activating mutation alleles ; *, stop codon.

with EGFR-TKI.[20,27-29] We previously showed that first-generation EGFR-TKI were effective for the treatment of tumors with a low T/A ratio determined by dPCR and that the T/A ratio increased markedly after the acquisition of EGFR-TKI resistance.[20] A high T/A ratio in tumor or plasma samples has also previously been associated with a high treatment efficacy of third-generation EGFR-TKI.[13,21,22] These previous data suggest that assessment of the T/A ratio allows more accurate evaluation of the contribution of T790M to resistance to first- or second-generation EGFR-TKI and improves the concordance between liquid biopsy and tumor biopsy for the detection of T790M. We therefore applied the T/A ratio to determination of T790M positivity in tumor and plasma samples by dPCR and NGS, resulting in an improvement in the specificity of plasma analysis compared with tumor analysis to 100% without a reduction in sensitivity (Figure 1B).

Our comprehensive assessment based on the T/A ratio indicated that T790M was positive in 13 (52.0%) of 25 patients at the time of systemic PD development (Table 4, Figure 2). Median PFS for treatment with afatinib was longer in patients with than in those without T790M at systemic PD (15.1 vs 10.9 months, $P = 0.25$; Figure 3B), consistent with previous findings.[26,30] Although median time to systemic PD was longer than median PFS in patients with T790M at systemic PD (17.9 vs 15.1 months), such a difference was not apparent in patients without T790M at this time (10.9 vs 10.9 months; Figure 3C). The observation that T790M-positive, TKI-resistant cells grow more slowly than T790M-negative cells in preclinical models[31] may have influenced the judgment of the investigators as to whether to continue the treatment with afatinib after radiological progression.

Although several mechanisms of resistance to second-generation EGFR-TKI including afatinib and dacomitinib have been identified in preclinical models,[32-35] such mechanisms other than the T790M mutation have rarely been detected in clinical samples. With the use of NGS, we examined genetic alterations in tumor or plasma samples obtained from 25 patients at the time of systemic PD development during afatinib treatment. CNG of *MET* (n = 1, 4%), CNG of *EGFR* concurrent with T790M (n = 1, 4%), CNG and the E545K mutation of *PIK3CA* concurrent with T790M of *EGFR* (n = 1, 4%), and CNG of *NRAS* (n = 1, 4%) were identified as putative mechanisms of resistance to afatinib among the 18 tumor samples (Table 4, Figure 2). Amplification of *MET* and the E545K mutation of *PIK3CA* have been implicated in resistance to first-generation EGFR-TKI, together accounting for ~5% of patients who acquired resistance to these drugs.[24,25] Amplification of *EGFR* concurrent with T790M has been implicated in resistance to the second-generation EGFR-TKI dacomitinib in a preclinical study[33] and was identified in plasma samples obtained from patients who acquired resistance to third-generation EGFR-TKI.[36] Amplification of *NRAS* has been implicated in resistance to the third-generation EGFR-TKI naquotinib in a preclinical model.[37] These previous data suggest that such genetic alterations constitute resistance mechanisms shared by various generations of EGFR-TKI. Resistance-related alterations coexisting with T790M of *EGFR* were identified in two patients of the present study, with such alterations having been implicated in reduced sensitivity to osimertinib.[19]

TABLE 4 Summary of identified putative mechanisms of resistance to afatinib (n = 25)

Patient	Activating mutation identified before afatinib treatment	Tumor samples (n = 18)			Plasma samples (n = 23)		Comprehensive assessment of T790M positivity	Putative resistance mechanisms other than T790M identified by NGS
		T/A by dPCR (%)	T/A by NGS (%)	ARMS ΔC_t value for T790M	T/A by dPCR (%)	T/A by NGS (%)		
1	p.E746_A750 del	**17.30**	**75.27**	**2.22**	0	0	Yes	
2	p.E746_A750 del	**48.25**	**62.30**	7.49	**120.00**	**17.70**	Yes	
3	p.E746_A750 del	**20.69**	**24.17**	**3.71**	**8.33**	**24.24**	Yes	
4	L858R	**24.32**	**20.37**	**5.17**	**50.00**	0	Yes	*PIK3CA*: p.E545K, *PIK3CA* CNG
5	L858R	**24.73**	**28.22**	**3.71**	**75.00**	**28.21**	Yes	
6	p.E746_A750 del	**50.00**	**39.67**	**3.67**	**33.33**	**42.77**	Yes	
7	p.S752_I759 del	NE[a]	**45.60**	**3.98**	NE[a]	0	Yes	
8	p.E746_A750 del	0	0	ND	0	0		
9	p.E746_A750 del	0	0	13.91	0	0		
10	p.E746_A750 del	1.52	0	13.11	0	0		
11	p.E746_A750 del	0	0	10.87	1.64	1.39		
12	p.E746_S752>V	NE[b]	0	ND	0	0		
13	p.E746_A750 del	1.03	5.53	13.08	0	0		*NRAS* CNG
14	p.E746_A750 del	1.09	0	ND	0	0		
15	L858R	0.16	0	ND	3.16	8.12		*MET* CNG
16	L858R	0	0	ND	0	0		
17	L858R	**57.83**	**68.61**	**2.32**	NA	NA	Yes	*EGFR* CNG
18	p.L747_P753>S	**15.00**	**59.74**	**4.84**	NA	NA	Yes	
19	L858R	NA	NA	NA	0	0		
20	p.E746_A750 del	NA	NA	NA	**50.00**	**77.98**	Yes	
21	p.E746_A750 del	NA	NA	NA	**150.00**	**12.15**	Yes	
22	p.E746_A750 del	NA	NA	NA	**58.33**	**70.86**	Yes	
23	p.E746_A750 del	NA	NA	NA	0.25	0.65		
24	L858R	NA	NA	NA	0	0		
25	L858R	NA	NA	NA	**31.25**	**41.5**	Yes	
T790M positivity		8 Yes (50.0%)	9 Yes (50.0%)	8 Yes (44.4%)	9 Yes (40.9%)	8 Yes (34.8%)	13 Yes (52.0%)	

T/A ratios >5.0% for dPCR and >10.0% for NGS as well as ΔC_t values <7.40 for ARMS are shown in bold. Discordant results for T790M positivity among ARMS and the T/A ratio for dPCR and NGS are shaded in gray.

[a]The T/A ratio was not evaluable (NE) because the activating mutation (p.S752_I759 del) is not recognizable by dPCR.

[b]The T/A ratio was not evaluable because the activating mutation seemed to disappear during afatinib treatment.

ARMS, amplification-refractory mutation system; CNG, copy number gain; dPCR, digital PCR; NA, not available; ND, not detected; NGS, next-generation sequencing; T/A ratio, number of T790M alleles to that of activating mutation alleles.

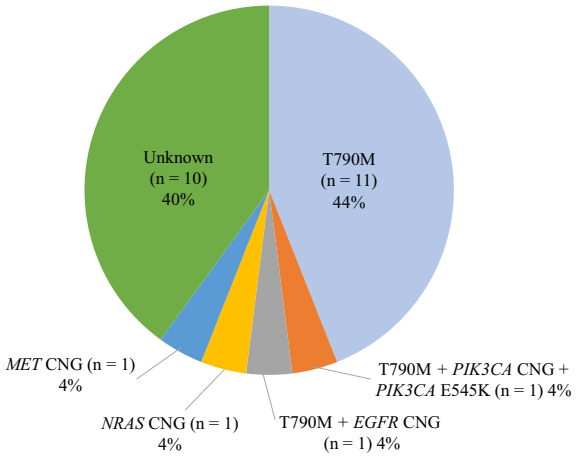

FIGURE 2 Frequency of putative mechanisms of acquired resistance to afatinib among the 25 patients for whom tumor or plasma samples were available at the time of systemic of progressive disease development. CNG, copy number gain; EGFR, epidermal growth factor receptor

Evaluation of alterations coexisting with *EGFR* T790M is thus important for assessment of the efficacy of subsequent treatment with osimertinib in patients who acquire resistance to first- or second-generation EGFR-TKI. Amplification of *HER2* and transformation to small-cell carcinoma, which are also implicated in resistance to first-generation EGFR-TKI, were not identified in the present study. The irreversible and pan-HER nature of inhibition by afatinib might give rise to a difference in resistance mechanisms compared with first-generation EGFR-TKI. The various resistance mechanisms identified in tumor samples of the present study were not detected by NGS in plasma samples obtained at systemic PD, possibly because we did not use an ultrasensitive method such as CAPP-Seq for library preparation.[19,36,38]

Mutations of *TP53* were frequently identified in plasma samples collected at the time of systemic PD development as well as in tumor samples (Table 3). In 9 (90.0%) of 10 patients for whom mutations of *TP53* were detected in plasma or tumor samples at systemic PD, the same type of *TP53* mutation was also detected in tumor or plasma samples obtained before initiation of afatinib treatment (Table 3).

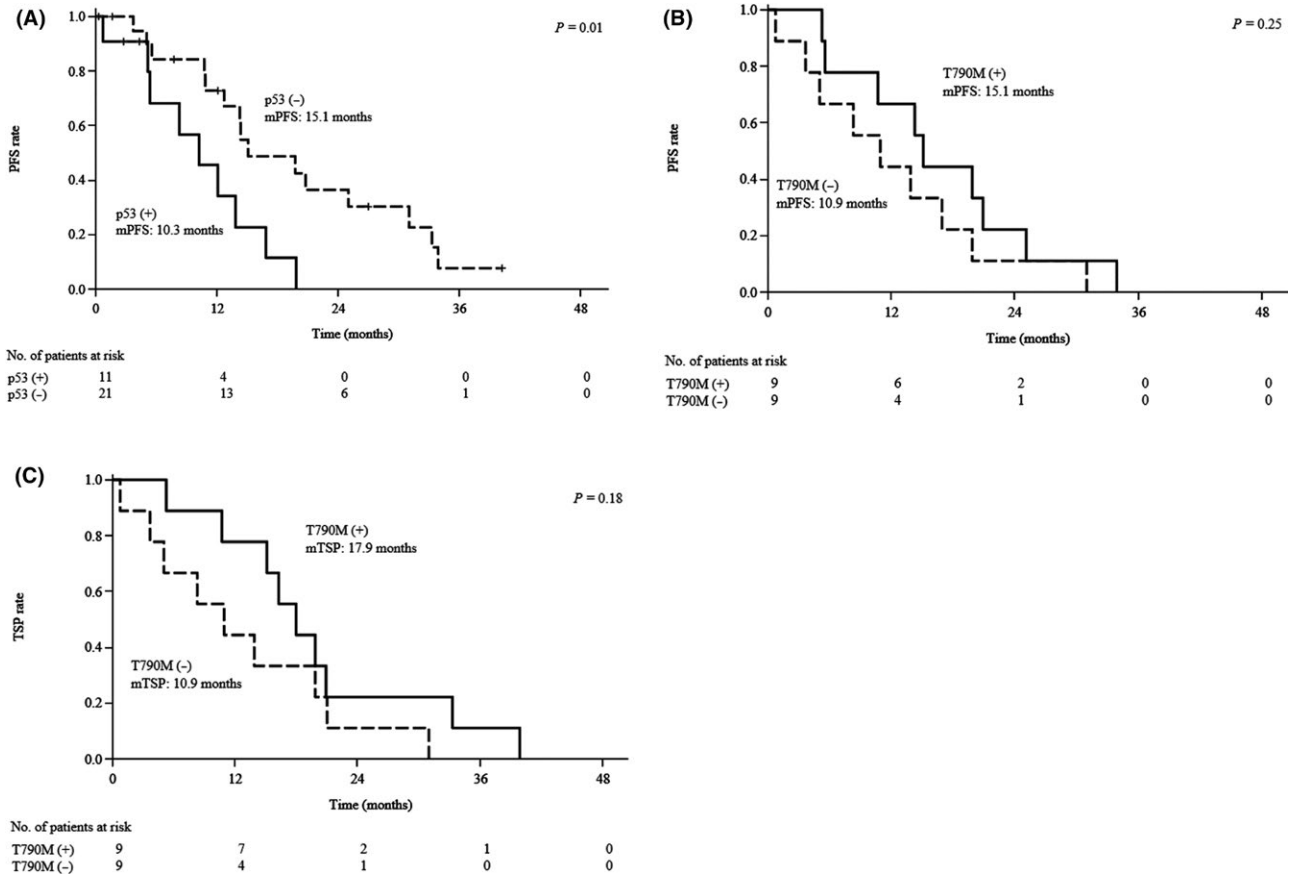

FIGURE 3 Survival analysis according to *TP53* or *EGFR* T790M status. A, Kaplan-Meier plots of progression-free survival (PFS) for the study subjects according to *TP53* mutation status in tumor samples obtained before afatinib treatment. B,C, Kaplan-Meier plots of PFS and time to systemic progressive disease (PD) (TSP), respectively, for the study subjects according to T790M status based on the T/A ratio of tumor samples at systemic PD development. Median (m) values for PFS and TSP, and the *P* value for the difference between each pair of curves as determined with the log-rank test, are indicated. EGFR, epidermal growth factor receptor; T/A ratio, number of T790M alleles to that of activating mutation alleles

Median PFS was significantly shorter in patients whose tumors harbored a *TP53* mutation before afatinib treatment than in those whose tumors did not (10.3 vs 15.1 months, $P = 0.01$; Figure 3A), consistent with previous findings.[39] All these mutations were located in exons 5-8 of *TP53*, which encode the DNA-binding domain that recognizes a consensus sequence in the promoters of several genes related to DNA repair and apoptosis.[40,41] The p53 protein has been shown to enhance gefitinib-induced apoptosis in NSCLC cells by upregulation of FAS, and *TP53* mutations reduce sensitivity to EGFR-TKI.[42,43] We previously showed that the allele frequency for *TP53* mutations and that for *EGFR*-activating mutations in plasma samples evaluated by NGS changed concordantly during afatinib treatment, with the frequencies declining during the period that afatinib was effective and increasing at disease progression.[16] These findings suggest that the presence of de novo coexisting mutations of *TP53* in plasma or tumor samples before EGFR-TKI treatment may be a biomarker that predicts a limited efficacy of such drugs for *EGFR* mutation-positive NSCLC. Coexisting minor mutations of *EGFR* that have been implicated in reduced efficacy of EGFR-TKI[44] were not detected in either tumor or plasma samples at baseline in the present study (data not shown).

There are several limitations to the present study. First, the sample size was small. Second, although the specificity of plasma analysis compared with tumor analysis was improved by the quantitative evaluation of T790M, the sensitivity of plasma analysis was still low. Given that false-negative results are a major problem for liquid biopsy, clinicians need to be careful in interpreting the findings of cfDNA analysis. Finally, the efficacy of subsequent treatment with osimertinib according to the value of the T/A ratio was not evaluated because the enrolment period for this prospective study was long before the approval of osimertinib and the cobas EGFR Mutation Test v2 (Roche Diagnostics, Risch-Rotkreuz, Switzerland), a companion diagnostic, in Japan. Further analysis based on the efficacy of osimertinib and application of this test is warranted to confirm the usefulness of the T/A ratio for determination of T790M positivity.

Next-generation sequencing analysis of tumor or plasma samples is already in clinical use for determination of tumor molecular profiles in precision medicine. Such analysis can identify genetic alterations that coexist with *EGFR*-activating mutations including a minor clone of the T790M mutation of *EGFR* and *TP53* mutations. Quantitative evaluation of T790M will thus be important for assessment of the contribution of this mutation to EGFR-TKI resistance. Further studies of the mechanisms of resistance to EGFR-TKI and of the pathways that limit the tumor response to these drugs are warranted to overcome the problems associated with both intrinsic and acquired drug resistance.

CONFLICTS OF INTEREST

D. Harada has received honoraria from AstraZeneca, Bristol-Myers Squibb, Eli Lilly Japan, Kyowa Hakko Kirin, Ono Pharmaceutical, and Yakult Honsha. K. Nosaki has received honoraria from AstraZeneca, Boehringer Ingelheim, Chugai Pharmaceutical, Eli Lilly Japan, Ono Pharmaceutical, Pfizer, and Taiho Pharmaceutical as well as research funding from MSD and Novartis. K. Hotta has received honoraria and research funding from AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Chugai Pharmaceutical, Eli Lilly Japan, MSD, Novartis, and Ono Pharmaceutical; honoraria from Nihon Kayaku and Taiho Pharmaceutical; and research funding from Astellas. M. Nishio has received honoraria and research funding from AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Chugai Pharmaceutical, Daiichi Sankyo Healthcare, Eli Lilly Japan, Merck Serono, MSD, Novartis, Ono Pharmaceutical, Pfizer, and Taiho Pharmaceutical as well as research funding from Astellas. T. Kurata has received honoraria from AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Chugai Pharmaceutical, Eli Lilly Japan, MSD, and Ono Pharmaceutical. H. Akamatsu has received honoraria from AstraZeneca, Boehringer Ingelheim, Chugai Pharmaceutical, Eli Lilly Japan, MSD, Novartis, Ono Pharmaceutical, Pfizer, and Taiho Pharmaceutical as well as research funding from MSD. K. Goto and K. Nishio have received honoraria and research funding from Boehringer Ingelheim. Y. Nakanishi has received honoraria from Boehringer Ingelheim. I. Okamoto has received honoraria from Boehringer Ingelheim as well as research funding from AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Chugai Pharmaceutical, Eli Lilly Japan, MSD, Ono Pharmaceutical, Pfizer, and Taiho Pharmaceutical. All other authors declare no potential conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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