Cancer Science
Japanese Cancer Association JCA
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Monitoring *EGFR* T790M with plasma DNA from lung cancer patients in a prospective observational study

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Key words

DNA, erbB-1, lung neoplasms, molecular targeted therapy, mutation

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Funding Information

Hanshin-Saga Collaborative Cancer Study Group: A Japanese non-profit organization in some parts supported from AstraZeneca.

This trial was registered with the UMIN Clinical Trials Registry: UMIN000005131.

Received July 22, 2015; Revised November 5, 2015; Accepted November 9, 2015

Cancer Sci **107** (2016) 162–167

doi: 10.1111/cas.12847

Use of plasma DNA to detect mutations has spread widely as a form of liquid biopsy. *EGFR* T790M has been observed in half of lung cancer patients who have acquired resistance to EGFR tyrosine kinase inhibitors (EGFR-TKI). Effectiveness of monitoring T790M via plasma DNA during treatment with EGFR-TKI has not been established as an alternative to re-biopsy. This was a prospective multicenter observational study involving non-small cell lung cancer patients carrying *EGFR* L858R or exon 19 deletions, treated with EGFR-TKI. The primary objective was to determine whether T790M could be detected using plasma DNA in patients with progressive disease (PD). T790M was examined using the mutation-biased PCR and quenching probe (MBP-QP) method, a sensitive, fully-automated system developed in our laboratory. Eighty-nine non-small cell lung cancer patients were enrolled from seven hospitals in Japan. Sequential examinations revealed T790M in plasma DNA among 40% of patients who developed PD. Activating mutations, such as L858R and exon 19 deletions, were detected in 40% of patients using plasma DNA, and either T790M or activating mutations were observed in 62%. Dividing into four periods (before PD, at PD, at discontinuation of EGFR-TKI and subsequently), T790M was detected in 10, 19, 24 and 27% of patients, respectively. Smokers, males, patients having exon 19 deletions and patients who developed new lesions evidenced significantly frequent presence of T790M in plasma DNA. Monitoring T790M with plasma DNA using MBP-QP reflects the clinical course of lung cancer patients treated with EGFR-TKI. Detection of T790M with plasma DNA was correlated with *EGFR* mutation type, exon 19 deletions and tumor progression. Re-biopsy could be performed only in 14% of PD cases, suggesting difficulty in obtaining re-biopsy specimens in practice. Monitoring T790M with plasma DNA reflects the clinical course, and is potentially useful in designing strategies for subsequent treatment.

Companion diagnostics has seen growing importance, especially for molecular targeted therapy such as EGFR tyrosine kinase inhibitors (EGFR-TKI) in lung cancer. Recently, a second generation EGFR-TKI, afatinib, evidenced a potent anti-cancer effect against lung cancer cells harboring T790M,[1] but the anti-cancer effect in a phase 2b/3 randomized trial was unsatisfactory considering the results of preclinical studies.[2] Because examination of biomarkers related to acquired resistance to EGFR-TKI was not performed in that trial, it is speculated that the patients included those with cancers possessing various mechanisms of acquired resistance to EGFR-TKI. Recently, third generation EGFR-TKI, such as AZD9291 and CO-1686, have shown potent anti-cancer effects in T790M positive non-small cell lung cancer patients.[3,4] Considering this evidence, examination of biomarkers is indispensable for accurate assessment of anti-cancer effects, and timely monitoring of genetic alterations is necessary for determining appropriate treatment.

The use of circulating free DNA to detect genetic alterations, so called "liquid biopsy," has spread worldwide.[5–9] For the purpose of molecular diagnosis before treatment, evaluation of genetic alterations should be comprehensive to pick up changes specific to each individual using deep sequencing or a multiplex system.[7–9] For monitoring therapeutic effects, we can focus on the specific loci of genetic changes such as T790M mutation. Because repeated examinations are needed

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for monitoring, the ideal detection system should be simple, automated and non-invasive.

We have developed a fully-automated sensitive mutation detection system, the mutation-biased PCR and quenching probe (MBP-QP) method, for detection of *EGFR* T790M mutations with plasma DNA.[10] The detection limit is two copies, and the sensitivity is 0.3%. Our retrospective study showed that T790M mutation was detected in plasma DNA in 53% of lung adenocarcinoma patients who acquired resistance to EGFR-TKI, and monitoring of T790M using MBP-QP was reflective of the clinical course in lung cancer patients treated with EGFR-TKI.[10] Based on these results, we proceeded to a prospective multicenter observational study. The purpose of this investigation was to determine whether T790M detection using MBP-QP with plasma DNA is useful for monitoring acquired resistance to EGFR-TKI, and to assess the possibility of using the method to predict efficacy of EGFR-TKI targeted to T790M.

Patients and Methods

Study population. This study, the Hanshin-Saga T790M (HASAT) study, was a prospective multicenter observational study. Patients were recruited from seven hospitals in Japan between 1 February 2011 and 29 February 2012. All hospitals belonged to the Hanshin-Saga Collaborative Cancer Study Group, a Japanese non-profit organization. Eligibility criteria were a diagnosis of non-small cell lung cancer confirmed by histological or cytological examination with exon 19 deletions and L858R in patients who were scheduled to start, or had already begun, treatment with EGFR-TKI. Patients had measurable or non-measurable diseases according to the Response Evaluation Criteria in Solid Tumors (RECIST).[11] Patients were not eligible to participate if T790M was detected in cancer tissues or cells before EGFR-TKI treatment. Ascertainment of *EGFR* mutations including T790M before entry into the study was performed in one of two commercial clinical laboratories, SRL (Tokyo, Japan) or Mitsubishi Chemical Medience Corporation (Tokyo, Japan), and was covered by medical insurance. Detection methods were cycleave PCR techniques and peptide nucleic acid-locked nucleic acid PCR clamp, respectively. All patients provided written informed consent.

Study design. Detection of T790M in plasma DNA was performed using the MBP-QP method, as described previously.[10] Briefly, 200 μL of plasma was subjected to DNA isolation using a QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany), and 4 μL of purified DNA water was applied to MBP-QP, a fully automated detection system. Negative and positive controls were added to each examination. The analyses were performed using i-densy®, and the areas under the mutation peaks were determined by the "idensy AreaAna®" software (ARKRAY, Kyoto, Japan). T790M positivity was declared if the area was 8.0 or more. Examinations of T790M in plasma DNA were performed before treatment with EGFR-TKI and every 4 months after the start of treatment. Chest CT and tumor markers were examined every 2 months. These examinations were also performed upon detection of progressive disease (PD) and at discontinuation of EGFR-TKI, and after two courses of post-chemotherapy, as well as 1 month after starting EGFR-TKI to check T790M at the peak response time. PD was evaluated by each investigator, and central review was carried out to confirm these evaluations. Re-biopsy was requested but not mandatory. T790M was examined by MBP-QP using tissue specimens obtained before treatment with EGFR-TKI and at the time of PD. The primary objective was to investigate whether T790M can be detected in plasma DNA at the time of PD, and the secondary objective was to confirm the correspondence between T790M levels in plasma and cancer specimens. A further exploratory objective was to verify the association between detection of T790M and the effect of EGFR-TKI. The criteria of T790M detection at the time of PD was that T790M should be detected from 30 days before to 70 days after PD considering the interval in which plasma samples were collected. Activating mutations, such as exon 19 deletions and L858R, were also examined in plasma DNA using the wild inhibiting PCR and quenching probe system (WIP-QP) and MBP-QP method, respectively, which were modified versions of those reported previously.[12] These examinations were performed using the same plasma DNA in which T790M was detected. In T790M negative cases, the samples were obtained at the time of PD for patients who acquired resistance to EGFR-TKI, and for non-PD cases the samples were those obtained at the end of follow up.

Statistical analysis. The association between T790M status in plasma DNA and clinical parameters was tested using the χ^2-test for categorical data and the *t*-test for continuous data. The survival rate was calculated according to the Kaplan–Meier method with differences assessed using the log rank test. Cox proportional hazards regression analysis was used to calculate the hazard ratio and the 95% confidence interval (CI) of the survival outcome of lung cancer patients. *P*-values are two-tailed; those below 0.05 were considered statistically significant. All statistical analyses were conducted using IBM SPSS Statistics 19 (SPSS, IBM, Tokyo, Japan).

Results

Patient characteristics. Out of 90 patients assessed for eligibility, 1 was excluded for lack of measurable and non-measurable lesions (Fig. S1). Characteristics of the 89 registered patients are shown in Table S1. The number of recruited patients having exon 19 deletions or L858R mutation was similar. Among the 89 patients, 83% had no history of EGFR-TKI treatment, whereas the remaining 17% had received prior EGFR-TKI. Although patients with cancer harboring T790M, which was examined in our own hospital, were excluded, T790M was detected in 5 of 77 samples (6.5%) that were available for re-examination using MBP-QP. Because 2 of the 89 patients died before plasma samples could be collected, 87 patients were analyzed. A total of 58 patients developed PD, in 23 of whom T790M was detected in plasma DNA. Dividing into two groups, with or without a history of EGFR-TKI treatment, T790M was detected in 18 of 47 patients (35%) among those who were EGFR-TKI naïve, and in 5 of 11 (45%) among those previously treated with EGFR-TKI. T790M was detected in 7 of 29 patients with non-PD. Re-examination of T790M in cancer tissue samples before EGFR-TKI treatment using MBP-QP revealed positivity in 5.9 and 7.7% among the patients whose diseases developed PD or not, respectively.

Profile of T790M detection with plasma DNA. T790M was detected in 23 of 58 patients (40%) whose diseases developed PD (Table 1). Dividing into four time periods (before PD, at PD, at discontinuation of EGFR-TKI and subsequently), T790M was detected in 10, 19, 24 and 27% of cases, respectively. T790M was first detected anywhere from 11 months before PD to 9 months after (Fig. S2): at PD in 39% (9/23), and at or after PD in 74% (17/23). Activating mutations were detected overall in 40% (35/87) of patients in this study; exon 19 deletions were detected in 41% (18/44) of patients and L858R in 40% (17/43).

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Table 1. Detection of T790M with plasma DNA among the patients who developed PD

Total	23/58 patients (40%)
Before PD	6/58† (10%)
at PD	10/54† (19%)
at discontinuation	11/45† (24%)
After discontinuation	10/37† (27%)

†The ratio was calculated as number T790M positive/number of samples obtained. PD, progressive disease.

Among the patients who acquired resistance to EGFR-TKI, both T790M positivity and activating mutations were detected in 22% (Table S2), and T790M positive only or activating mutation only were detected in 17 and 22%, respectively. The proportion with either T790M positive or activating mutation was 62%. According to other work, T790M was detected, but activating mutations sometimes disappeared with re-biopsy specimens isolated from the patients who acquired resistance to EGFR-TKI.[13] These results suggest that a clone of cancer cells carrying T790M exists after acquired resistance. The profiles of T790M positive patients who developed PD are shown in Figure 1. T790M was detected once in 16 patients and twice or more in 7 patients. Among the former, T790M was mostly detected after PD in 13 of 16 patients. For Patient No. 7, T790M was also detected in the cancer specimens before EGFR-TKI treatment using MBP-QP. Because the relative amount of mutant allele can be estimated by determination of the area under the mutation peak, we evaluated the relationship between the amount of mutant and clinical course (Fig. S3). In the cases in which T790M was detected twice or more, the area under the mutation peak tended to be chronologically increasing. Comparing PD and non-PD, the amount of mutant allele tended to be higher in PD, except in 1 patient, No 28. Among the patients with non-PD, T790M was detected in 7 patients (Fig. S4). T790M was positive at the end of the follow-up period in 3 patients, and it was repeatedly detected in 1 patient. In Patient No. 30, T790M was repeatedly detected at the end of the follow-up period, at which time T790M was also detected in the primary lesion, suggesting that the T790M positive clone expanded.

Concordance of T790M detection between plasma DNA and re-biopsy specimens. We obtained eight re-biopsy specimens, including primary lesions from 3 patients, intrapulmonary metastases from 3 patients and pleural effusions from 2 patients (Table 2). T790M was detected in 4 and 2 patients from re-biopsy and plasma DNA, respectively. As for the case in which T790M was positive in both samples, re-biopsy was taken from the primary lesion. The samples positive for T790M only in re-biopsy were from one primary lesion and two intrapulmonary metastatic lesions, and the case that was positive for T790M only in plasma DNA was for a re-biopsy from pleural effusion.

Clinical characteristics of patients with progressive disease: Comparing T790M positive and negative patients. T790M was more frequently detected in males, smokers and patients with exon 19 deletions (Table 3). Moreover, T790M was detected frequently when new lesions developed, but less frequently in the case of mere exacerbation of target lesions such as primary lesions and/or lymph node metastasis. Two of three T790M positive patients who developed PD manifesting as an increase in target lesions showed enlargement of metastatic lesions in the liver or adrenal gland. These results indicate that detection of T790M in plasma DNA was related to distant metastasis. Comparing overall survival (OS) between patients who were T790M positive versus negative among those who developed PD, the group negative for T790M evidenced significantly prolonged OS (median, 516 days *vs* 782 days; hazard ratio for death with T790M positive in plasma DNA, 2.15; 95% CI, 1.11–4.14; $P = 0.020$) (Fig. 2a). Combined analysis of T790M and activating mutations with plasma DNA showed that having neither T790M nor activating mutations was associated with better prognosis than having both T790M and activating mutation or either alone (median, 807 days *vs* 509 days; hazard ratio for death with neither T790M nor activating mutation in plasma DNA, 0.240; 95% CI, 0.104–0.553; $P = 0.010$) (Fig. 2b).

Discussion

In this prospective study, we observed the following. First, monitoring T790M in plasma DNA using MBP-QP is feasible, and it reflects the clinical course of lung cancer patients with L858R or exon 19 deletions treated with EGFR-TKI. Second, detection of T790M with plasma DNA is not correlated with the effect of EGFR-TKI, but with *EGFR* mutation type,

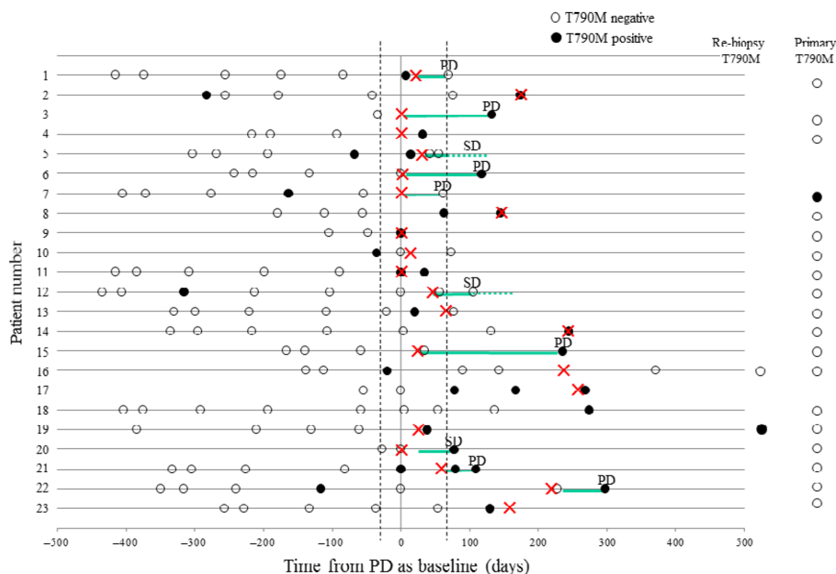

Fig. 1. Monitoring T790M with plasma DNA among patients who developed progressive disease (PD). Serial analysis of T790M with plasma DNA among patients with PD is shown. Time is time from PD as baseline. Open circles and filled circles indicate T790M negative and positive, respectively. Our criterion of PD was from 30 days before to 70 days after PD (dotted line) considering that blood collection was every 4 months. ×, discontinuation of EGFR-TKI; PD, progressive disease as tumor response of chemotherapy; primary T790M, T790M status in primary lesions; re-biopsy T790M, T790M status in re-biopsy specimens; SD, stable disease as tumor response of chemotherapy. Green line: period of chemotherapy.

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Table 2. Concordance between plasma DNA and re-biopsy

Age	Gender	Smoking	*EGFR* mutation	Effect of EGFR-TKI		T790M		
				Best response	Reason for PD	Plasma	Re-biopsy	Site of re-biopsy
86	Female	Never	Exon 19	PD	Pleural effusion	Negative	Negative	Pleural effusion
59	Female	Never	Exon 19	PR	Primary lesion	Negative	Negative	Primary lesion
70	Female	Smoker	Exon 19	PR	New lesion	Positive	Positive	Primary lesion
69	Male	Never	Exon 19	SD	New lesion	Negative	Negative	Lung metastasis
65	Female	Smoker	L858R	SD	Primary lesion	Negative	Positive	Primary lesion
81	Male	Smoker	L858R	SD	Pleural effusion	Positive	Negative	Pleural effusion
66	Male	Smoker	L858R	PR	New lesion	Negative	Positive	Lung metastasis
74	Female	Never	L858R	NE	Non-PD	Negative	Positive	Primary lesion

NE, not evaluated; PD, progressive disease; PR, partial response; SD, stable disease

exon 19 deletions and tumor progression. Finally, re-biopsy can be performed in only a small fraction of patients with PD, and concordance with re-biopsy is approximately half.

T790M was detected in plasma DNA in 40% of the 58 patients whose diseases developed PD. Activating mutations were detected in 40%, and either T790M or activating mutations was present in 62%, suggesting that tumor-derived DNA was contained in peripheral blood of at least 62% of the study population. Monitoring T790M with plasma DNA showed that frequency of T790M detection increased following the onset of PD. The first detection period was mostly clustered around the time of PD and afterwards, and the area under the mutation peak increased with the passage of time, indicating that the results of T790M reflect the clinical course in the population. In addition, T790M was more frequently detected in the PD patients with previous EGFR-TKI treatment compared to those who were EGFR-TKI naïve, suggesting that T790M appears in plasma as the tumor progresses. When T790M was sporadically detected before PD, the amount of T790M estimated by the area under the mutation curve was relatively small. These data suggest that fluctuation in the detected level of T790M occurs because of the low copy number in peripheral blood.

Table 3. Comparison between T790M positive and negative among the patients who developed PD

	T790M in plasma		*P*
	Positive (N = 23)	Negative (N = 35)	
Age (median; range)	70 (54–89)	68 (48–88)	NS
Gender (%)			
Female	12 (32)	26 (68)	0.04
Male	11 (55)	9 (45)	
Smoking status (%)			
Non-smoker	10 (27)	27 (73)	<0.01
Smoker	13 (62)	8 (38)	
EGFR mutation (%)			
Exon 19 deletions	16 (52)	15 (48)	0.05
L858R	7 (26)	20 (74)	
Best response (%)			
PR or SD	20 (38)	32 (62)	NS
PD	3 (50)	3 (50)	
Duration of TKI treatment	400 days	393 days	NS
Median PFS	303 days	344 days	NS
Reasons for PD (%)			
New lesions	12 (52)	11 (48)	0.01
Increase in target lesions	3 (15)	17 (85)	

Patient characteristics with or without T790M in plasma were compared using the χ^2-test for categorical data and the *t*-test for continuous data. NS, not significant; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease; TKI, tyrosine kinase inhibitor.

According to previous findings, T790M existing *in cis* with activating mutations such as L858R and exon 19 deletions induced transforming activity *in vitro* and led to tumor formation in animal experiments, suggesting that T790M has oncogenic activity.[14] In the clinical situation, lung cancer patients who were T790M positive in re-biopsy showed better prognosis compared to those who were T790M negative,[13,15] suggesting that T790M positive clones may be less aggressive. However, in a retrospective study, we reported that T790M positivity in plasma DNA showed poorer prognosis compared to T790M negativity.[10] That result was confirmed in the present work. We showed also in the present study that the detection rate and amount of T790M increased as tumors developed. In addition, T790M was frequently detected when new lesions, mostly distant metastases, appeared. Combined analysis of T790M and activating mutations showed that the presence of either mutation (or both) resulted in poor prognosis. Considering all of these findings together, T790M itself does not seem to have strong metastatic potential, but the appearance of DNA derived from tumor in peripheral blood is correlated with tumor progression, including metastasis. Tumor-derived DNA has been thought to be released into peripheral blood after necrosis and/or apoptosis, but mainly necrosis, and it has been assumed to be related to tumor burden, stage and cellular turnover.[16,17] Recent data from animal studies in our laboratory indicate that the amount of tumor-derived DNA is related to aspects of tumor progression, such as metastasis.[18]

The effects of EGFR-TKI, such as best response, duration of treatment and progression-free survival, were not correlated with detection of T790M in plasma. In contrast, T790M in plasma was frequently observed in smokers, males and patients with exon 19 deletions. These results suggest that appearance of T790M in plasma is related to biological characteristics of lung cancer, but not status of treatment with EGFR-TKI. T790M was detected more frequently in lung cancers carrying exon 19 deletions even with re-biopsy.[19] According to a previous report, biological characteristics differed between exon 19 deletions and L858R when T790M coexisted with each mutation.[14] Catalytic and tumorigenic activity with a combination of T790M with exon 19 deletions was more aggressive than that with L858R. Formation of tumors carrying T790M and exon 19 deletions was more rapidly growing than

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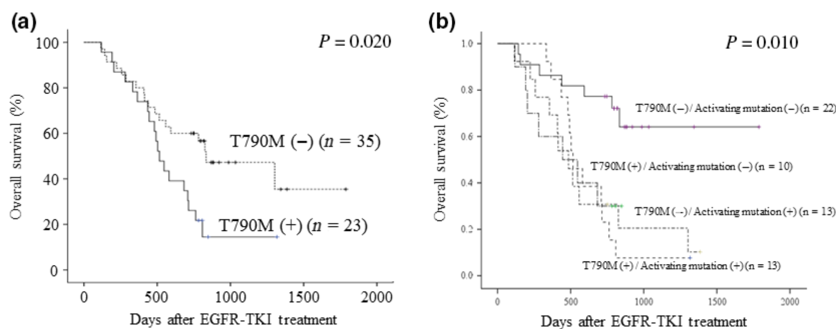

Fig. 2. Overall survival among patients with progressive disease (PD). Kaplan–Meier curve estimate of overall survival in patients with PD divided into two or four groups. (a) Two groups: T790M positive and negative. (b) Four groups: T790M positive and activating mutation positive, T790M positive and activating mutation negative, T790M negative and activating mutation positive, and T790M negative and activating mutation negative. *P*-values were calculated using the log rank test.

T790M and L858R in the animal model. Considering that appearance of T790M in plasma is correlated with tumor progression, it is plausible that coexistence of T790M with exon 19 deletions is observed more frequently than coexistence of T790M with L858R.

Investigating the concordance between plasma and re-biopsy was the secondary objective of the present study. However, re-biopsy samples were obtained from only 14% of patients, all from intra-thoracic regions. This suggests that re-biopsy is difficult to perform, especially from distant metastatic sites. Indeed, reports from other laboratories show that most re-biopsy is performed from intra-thoracic lesions, including the enlarging primary lesion, intra-pulmonary lesion and pleural effusion.[15,20–22] Recently, inter-tumor and intra-tumor heterogeneity has been reported.[23,24] In addition to different genomic alterations between primary and metastatic lesions, spatial variation in the primary lesion has also been reported. Investigation of T790M and MET amplification in each primary and metastatic lesion after acquired resistance revealed inconsistencies, suggesting that inter-tumor heterogeneity occurs after treatment with EGFR-TKI.[24] Although re-biopsy has been the gold standard to assess the mechanism of acquired resistance to EGFR-TKI, these reports indicate that a single biopsy cannot reflect genomic alterations throughout the tumors in each patient. A recently published paper showed that the concordance between re-biopsy and liquid biopsy including plasma DNA and circulating tumor cell was 57–60%.[25] Based on evidence from whole-genome sequencing, somatic mutations in plasma DNA were detected from 21 to 55% of mutations detected in corresponding tumor DNA. The detection rate of mutations observed in the primary lesion was low, while that of metastatic lesions was high in plasma DNA.[26] These results indicate that detection of mutations in plasma DNA reflects approximately half of the mutations occurring in tumors, and inter-tumor and intra-tumor heterogeneity may be the cause of disagreement in mutation status between re-biopsy and liquid biopsy.

Monitoring of T790M in plasma DNA could assist us in designing strategies for subsequent treatment. To confirm that plasma DNA reflects dominant genomic alterations, it is necessary to investigate the anti-cancer effect of T790M inhibitors using detection of T790M with plasma DNA as a biomarker.

Acknowledgments

This study was supported by the Hanshin-Saga Collaborative Cancer Study Group, a nonprofit organization partly supported by AstraZeneca. We thank the patients and their families for their support and participation in this trial. We are also grateful to the data managers and other support staff of the Hanshin-Saga Collaborative Cancer Study Group, especially Mitsuho Edagawa and Akemi Sato.

Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Trial profile.

Fig. S2. First detection of T790M with plasma DNA among patients whose diseases developed PD.

Fig. S3. Amount of T790M in plasma DNA estimated by area under the mutation peak.

Fig. S4. Monitoring of T790M in plasma DNA among patients whose diseases did not develop progressive disease (PD).

Table S1. Characteristics of patients.

Table S2. Detection of T790M and activating mutations with plasma DNA among patients who acquired resistance to EGFR tyrosine kinase inhibitors (EGFR-TKI).

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