

Plasma T790M and HGF as potential predictive markers for EGFR-TKI re-challenge

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Abstract. Re-challenge with epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) has been suggested to potentially improve survival in certain populations of patients with advanced lung cancer, but predictive markers for the success of EGFR-TKI re-challenge have not been identified. The present study analyzed 16 re-challenges with EGFR-TKI undertaken in 12 patients with lung adenocarcinoma by investigating T790M and hepatocyte growth factor (HGF) in plasma coupled with clinical characteristics. *EGFR* mutations in plasma DNA were detected using the wild inhibiting PCR and quenched probe system for exon 19 deletions, and T790M and L858R were detected using the mutation-biased PCR and quenched probe system. HGF levels in the plasma were measured by enzyme-linked immunosorbent assay, and the ratio of HGF levels prior to re-challenge to those prior to the previous EGFR-TKI treatment was calculated. Two re-challenges demonstrated partial response, six remained as stable disease and eight had progressive disease (PD). A total of 4 of the 5 patients with a history of T790M positivity based on plasma DNA levels had PD. A total of 7 of the 8 patients who had ≥ 1.5 -fold elevation of HGF prior to re-challenge with EGFR-TKI suffered PD. Elevation of the HGF ratio to ≥ 1.5 was significantly associated with poor response to EGFR-TKI re-challenge. Having no history of T790M and an HGF ratio < 1.5 was significantly associated with a positive response to EGFR-TKI re-challenge. A combination of T790M detection and HGF quantification using plasma is a potentially useful assay system for predicting the effect of EGFR-TKI

re-challenge. Future prospective studies are required to confirm the predictive validity of these markers.

Introduction

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) have produced dramatic anti-cancer effects in patients with non-small cell lung cancer (NSCLC) carrying *EGFR* activating mutations (1-3). The first generation of EGFR-TKIs, including gefitinib and erlotinib, conferred significantly prolonged progression-free survival (PFS) in these patients. The second generation of these drugs, afatinib, brought a remarkable prolongation of overall survival, up to 33 months, in particular in patients with exon 19 deletions (4). In spite of the effectiveness of EGFR-TKIs, patients eventually acquire resistance. Several treatment strategies have been evaluated in clinical trials and practice following the onset of acquired resistance. First, agents targeted at molecules contributing to acquired resistance have been considered. Based on mechanisms including the secondary *EGFR* mutation, T790M, MET proto-oncogene, receptor tyrosine kinase (MET) amplification, and hepatocyte growth factor (HGF) overexpression, second and third generation EGFR-TKIs and MET inhibitors have been developed (5-9). Afatinib confers a potent anti-cancer effect against lung cancer cells harboring T790M, but a phase 2b/3 randomized trial revealed that the overall response rate and PFS of patients with lung cancer who were previously treated with EGFR-TKI were 7% and 3.3 months, respectively, which was not satisfactory considering the results of preclinical studies (9). Since examination of biomarkers associated with acquired resistance to EGFR-TKI was not performed in that trial, it was speculated that the patients included those with cancers possessing various mechanisms of acquired resistance to EGFR-TKI. The T790M inhibitor AZD9291 has proceeded to clinical trials and significant anti-cancer efficacy has been demonstrated in T790M-positive lung cancer patients, with an overall response rate and PFS of 61% and 9.6 months, respectively (10,11). MET inhibitors including anti-MET antibody and MET-TKI require predictive markers for the selection of the appropriate population according to the results of clinical trials (12-14).

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The second strategy for acquired resistance is re-challenge with EGFR-TKI. Re-challenge with gefitinib or erlotinib, often subsequent to cytotoxic treatment following initial EGFR-TKI, may prolong survival for patients with advanced lung cancer who previously achieved a positive response to EGFR-TKI. A retrospective study revealed that OS was significantly longer in the patients who underwent gefitinib re-challenge compared with those who did not undergo re-challenge (15). When evaluation of the efficacy of EGFR-TKI re-challenge was limited to patients who achieved good control of disease with the first line of EGFR-TKI, disease control rate and PFS were 56-73% and 3.4-5.6 months, respectively (16-21). On the other hand, patients whose disease was not be controlled with the first line of EGFR-TKI tended to exhibit poor efficacy with re-challenge (19,20,22-24). From these results, the effect of first EGFR-TKI may be considered a predictive marker of efficacy of re-challenge, but specific molecular markers associated with mechanisms of acquired resistance have not been identified.

Examination of biomarkers is indispensable for accurate assessment of anti-cancer effects. However, re-biopsy is difficult because it is an invasive procedure for elderly patients with lung cancer and poor lung function. In addition, tumor biological characteristics change frequently, and monitoring of genetic alterations is required to decide treatment (25-27). Therefore, non-invasive liquid biopsy using peripheral blood, which it is possible to repeatedly perform, is a preferable method for monitoring biomarkers. Our group previously developed a fully automated T790M monitoring system using circulating plasma DNA, mutation-biased polymerase chain reaction (PCR) and quenched probe (MBP-QP) system (28). As the system utilizes peripheral blood, it is possible to examine T790M repeatedly. Our previous retrospective study using the MBP-QP system demonstrated that T790M was detected in 53-56% of patients who acquired resistance to EGFR-TKI (28,29). In addition, a prospective multicenter observational study was then performed to determine whether T790M detection using MBP-QP system with plasma DNA was useful for monitoring acquired resistance to EGFR-TKI, and T790M was reproducibly detected in 40% of patients whose disease became progressive (30). HGF levels in the plasma were also determined, and an elevation of HGF of ≥ 1.5 -fold was observed in 38% of the population (29). A combination of T790M detection and HGF quantification using plasma revealed that T790M and/or elevation of HGF were detected in 69% of that population.

The present study investigated whether detection of these molecular markers would be useful for determining the appropriate population for re-challenge with EGFR-TKI. In order to administer various EGFR-TKIs appropriately, it is important to determine what clinical characteristics and biomarkers are predictive of treatment efficacy.

Materials and methods

Patient selection. Plasma samples were obtained from 225 patients with lung cancer treated at Saga University Hospital (Saga, Japan) between January 2000 and October 2013. Among these patients, 60 were treated with EGFR-TKI and 12 adenocarcinoma patients underwent a total of 16 re-challenges with

EGFR-TKI (re-challenge was performed on the same patients 1-3 times). The clinical features of the patients that underwent re-challenge are listed in Table I. Plasma samples were repeatedly collected throughout the course of treatment. Clinical stage of the cancer was determined according to criteria in the 7th edition of the International Union Against Cancer at the times plasma samples were obtained (31). The study protocol was approved by the Clinical Research Ethics Committees of Saga University (Saga, Japan). All patients provided informed consent for blood and tissue specimen collection and genomic testing, according to the Declaration of Helsinki.

DNA extraction from plasma for detection of EGFR mutations. Peripheral blood samples were collected into tubes containing 3.8% citric acid. Plasma was immediately separated centrifugation at 1750 x g at 4°C for 20 min. Supernatants were collected and stored at -80°C until assays were performed. DNA was isolated from 200 μ l patient plasma using a QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. DNA was eluted with 50 μ l ultrapure water, and 4 μ l was applied for detection of EGFR mutations as described in the next section.

Detection of EGFR mutations. Exon 19 deletions and point mutations including L858R and T790M were detected by the wild inhibiting PCR and quenched probe (WIP-QP) system and the MBP-QP system, respectively. These systems were fully automated using *i*-densy™ IS-5320 (ARKRAY Inc., Kyoto, Japan), as described previously (28,30,32). Briefly, WIP-QP consisted of wild inhibiting PCR (WIP) and quenched probe (QP) systems, as described previously (32). Wild inhibitor nucleic acid (WI) is complementary to the wild type sequence corresponding to the deletion part. WI suppresses amplification of the wild type sequence by binding to the wild-type template but not the mutant, resulting in preferential amplification of the mutant sequence. The presence of deletions in amplified sequences was determined by monitoring the fluorescence intensity of a TAMRA-conjugated, guanine-specific quench fluorophore probe (QProbe, J-Bio21 Center, Tokyo, Japan), which was complementary to the wild type sequence containing the deletion part. Fluorescence intensity was measured at different temperatures to identify wild-type and mutant amplicons, and quantified as previously described (32). MBP-QP for the detection of L858R and T790M consisted of mutation-biased PCR (MBP) and quenched probe (QP) systems, with conditions used as previously described (28,32). For MBP, the primers for wild-type and mutant were mixed with genomic DNA, which results in high specificity as each primer competitively hybridizes to wild type and mutant sequences. In addition, the length of the reverse primer for mutant was longer than that for wild-type and the annealing temperature was designed to be optimum to mutant primer, resulting in higher efficiency of amplification of the mutant sequence. Detection was performed using the QP-system as with WIP-QP, as previously described (28,32).

Quantification of HGF plasma levels. HGF plasma levels were measured by enzyme-linked immunosorbent assay (Immunis HGF EIA; product code 1EH1; B-Bridge International, Inc., Mountain View, CA, USA; limit of detection, 100 pg/ml), as

Table I. Patient characteristics.

Patient no.	Re-challenge no.	Age	Sex	SI	EGFR activating mutation	Previous EGFR-TKI treatments		Chemotherapy between EGFR-TKI treatments		
						Effect of therapy	PFS (days)	Frequency	Optimal effect	TKI-free interval (days)
1	1	77	F	0	exon19	PR	301	1	PD	101
2	2	44	F	100	exon19	PR	348	2	SD	447
	3					PR	78	0	NA	3
3	4	40	M	200	L858R	PR	401	0	NA	1
	5					SD	81	2	SD	618
4	6	40	M	600	exon19	PR	322	2	SD	244
5	7	64	M	800	exon19	SD	493	2	SD	269
6	8	55	M	480	exon19	PR	734	2	PR	396
7	9	56	F	0	exon19	SD	509	1	SD	290
	10					SD	77	1	PR	376
	11					SD	149	2	SD	337
8	12	57	F	0	L858R	PR	361	2	PR	533
9	13	78	F	0	L858R	PR	259	1	SD	185
10	14	50	F	50	exon19	PR	377	3	SD	337
11	15	58	M	900	exon19	PR	420	1	PR	240
12	16	58	M	0	L858R	PR	269	1	SD	266

SI, smoking index; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; PFS, progression free survival; PD, progressive disease; PR, partial response; SD, stable disease; NA, not available; F, female; M, male.

described previously (30). A total of 50 µl plasma was applied to the assay system. All samples were assayed in duplicate. Color intensity was measured at 450 nm with a spectrophotometric plate reader (ARVO™ MX 1420 Multilabel Counter; PerkinElmer Inc., Waltham, MA, USA). HGF concentrations were determined by comparison with standard curves.

Statistical analysis. The associations between the response to EGFR-TKI re-challenge, plasma biomarkers and clinical characteristics were tested using the Fisher's exact test for contingency tables and the Mann-Whitney U test for continuous data. Statistical analyses were conducted using IBM SPSS 22 software (IBM SPSS, Armonk, NY, USA).

Results

Patient characteristics. Table I lists the clinical characteristics of the patients in this study. Re-challenges were performed 16 times on 12 patients in total: 3 times on 1 patient, 2 times on 2 patients, and 1 time on 9 patients, all diagnosed with adenocarcinoma. The ages of the patients ranged from 40-77 years (median age 57 years), there were 6 females (50%) and 5 non-smokers (42%). EGFR activating mutation was detected in the primary tumors of all patients: 8 patients (67%) had exon 19 deletions and 4 patients (33%) harbored the L858R mutation. Prior to the 16 re-challenges, 11 patients achieved partial response (PR) to EGFR-TKI treatment (69%) and 5 patients exhibited stable disease (SD; 31%; Table I). All patients had EGFR-TKI discontinued due to disease progression. Cytotoxic

chemotherapy including platinum, pemetrexed, bevacizumab, and taxans was administered in 1-3 regimens prior to re-challenge with EGFR-TKI on 14 occasions. PR and SD were seen as the optimal response in 4 and 9 instances, respectively (Table I). The TKI-free interval prior to EGFR-TKI re-challenge ranged from 1 to 618 days. The median EGFR-TKI free interval was 333 days in patients whose effect of EGFR-TKI re-challenge was PR or SD and 242 days in patients whose effect was PD (Tables I and II).

Biomarker analysis with plasma samples. Plasma samples were collected repeatedly during the treatment, including prior to and at the time of PD to previous EGFR-TKI treatment as well as prior to re-challenge (Table III). Prior to the previous EGFR-TKI treatment, T790M was not detected in any patients. Following PD, plasma DNA T790M turned to positive in four re-challenges following the previous EGFR-TKI. Among them, T790M disappeared in three re-challenges following treatment with cytotoxic chemotherapy following the previous EGFR-TKI. In 1 patient, T790M appeared following cytotoxic chemotherapy concomitant with tumor progression. T790M was continually observed from the time of PD following the previous EGFR-TKI to prior to re-challenge in 1 patient. EGFR activating mutations were detected with plasma DNA in all but 2 samples that were T790M positive.

Plasma HGF levels ranged from 39-394 pg/ml prior to previous EGFR-TKI and 39-680 pg/ml prior to re-challenge; with a median of 144 and 147.5 pg/ml, respectively. The change of HGF level in plasma prior to and following previous EGF-TKI

Table II. Comparison between effect of EGFR-TKI re-challenge and clinical parameters including biomarkers (n=16).

Parameter	Effect of EGFR-TKI re-challenge		P-value ^a
	PR+SD, n (%)	PD, n (%)	
Effect of previous EGFR-TKI treatment			0.500
PR	5 (45.5)	6 (54.5)	
SD	3 (60.0)	2 (40.0)	
PFS of previous EGFR-TKI treatment			0.285
≥6 months	7 (58.3)	5 (41.7)	
<6 months	1 (25.0)	3 (75.0)	
Effect of chemotherapy			0.285
PR	3 (75.0)	1 (25.0)	
SD, PA, NA	5 (41.7)	7 (58.3)	
TKI-free interval			0.248
Median (day)	333	242	
EGFR activating mutation (plasma DNA)			0.690
+	4 (50.0)	4 (50.0)	
-	4 (50.0)	4 (50.0)	
History of T790M (plasma DNA)			0.141
+	7 (63.6)	4 (36.4)	
-	1 (20.0)	4 (80.0)	
HGF ratio ^b			0.009
<1.5	6 (85.7)	1 (14.3)	
≥1.5	1 (12.5)	7 (87.5)	
Combination of T790M and HGF			0.001
Neither T790M nor HGF	7 (100.0)	0 (0.0)	
T790M ^c and/or HGF	1 (11.1)	8 (88.8)	

^aThe exact P-value based on the Fisher's exact test for contingency tables and the Mann-Whitney U test for continuous data. ^bHGF ratio is calculated by plasma HGF prior to previous EGFR-TKI treatment/plasma HGF prior to re-challenge. ^cT790M, history of T790M; HGF, elevation of HGF ratio ≥1.5. EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; PD, progressive disease; PR, partial response; SD, stable disease; PFS, progression free survival; NA, not available; HGF, hepatocyte growth factor.

and prior to re-challenge were investigated (Fig. 1). HGF levels decreased in cases that experienced effective re-challenge, whereas HGF levels were elevated in cases where re-challenge was ineffective. The ratio of HGF level prior to re-challenge to that prior to previous EGFR-TKI treatment ranged from 0.4-3.2, and 8 patients had a ≥1.5-fold elevation of HGF (Table III). When these ratios of plasma HGF level were calculated in the 12 patients who remained sensitive to EGFR-TKI treatment or who had EGFR-TKI discontinued due to side effects, they ranged from 0.2-1.4.

Response to re-challenge with EGFR-TKI. A total of 2 re-challenges demonstrated PR (12.5%) and 6 remained SD (37.5%), disease control rate was 50%, and the median PFS was 61 days (Table III). A total of 7 out of 8 patients with PD to re-challenge demonstrated a ≥1.5-fold elevation of the HGF ratio. Out of the 5 patients with a history of T790M positivity in plasma DNA, 4 had PD. A total of 3 out of the 4 patients who achieved PR as the optimal response with cytotoxic chemotherapy between EGFR-TKI treatments benefited from EGFR-TKI re-challenge.

Clinical characteristics and plasma biomarkers associated with the optimal response to EGFR-TKI re-challenge were analyzed (Table II). The effect of previous EGFR-TKI (optimal response as well as duration of treatment), optimal response to chemotherapy, TKI-free interval and detection of EGFR activating mutation in plasma all failed to evidence a significant association with the effect of re-challenge. An elevation of ≥1.5-fold in the HGF ratio was significantly associated with poor response to EGFR-TKI re-challenge (P=0.009). No history of T790M in the plasma and an elevation of the HGF ratio <1.5 together were significantly associated with a positive response to EGFR-TKI re-challenge (P=0.001).

A representative serial analysis of biomarkers for patient 2 is depicted in Fig. 2. The first treatment with the EGFR-TKI gefitinib demonstrated PR within 348 days of PFS. T790M and HGF elevation were not observed in plasma collected prior to this. Two regimens of chemotherapy were subsequently performed, and the outcomes were SD. Prior to the first re-challenge with erlotinib, neither T790M nor elevation of HGF ratio was observed, resulting in PR to the treatment.

Table III. Biomarkers and effects of EGFR-TKI re-challenge.

Re-challenge no.	T790M		EGFR-TKI re-challenge		EGFR-TKI re-challenge	
	Previous EGFR-TKI treatment		Prior to PD	HGF ratio ^b	Effect	PFS (days)
	Prior to PD	At time of PD				
1	-	-	- ^a	1.7	PD	22
2	-	-	- ^a	0.4	PR	78
3	-	-	- ^a	3.2	PD	34
4	-	-	- ^a	0.6	SD	81
5	-	-	-	1.9	PD	16
6	-	+	-	2.3	PD	26
7	-	-	- ^a	0.6	SD	60
8	-	+	-	0.5	PR	95
9	NA	-	- ^a	NA	SD	77
10	-	-	-	0.5	SD	149
11	-	+	-	1.5	PD	40
12	-	-	-	1.7	SD	138
13	-	-	- ^a	1.5	PD	25
14	-	-	+	1.7	PD	44
15	-	+	+	1.4	PD	18
16	-	-	- ^a	0.6	SD	95

^aSame samples as those obtained following previous EGFR-TKI treatment. ^bHGF ratio, plasma HGF prior to previous EGFR-TKI treatment/plasma HGF prior to re-challenge. EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; PD, progressive disease; HGF, hepatocyte growth factor; PFS, progression free survival; PR, partial response; SD, stable disease; NA, not available.

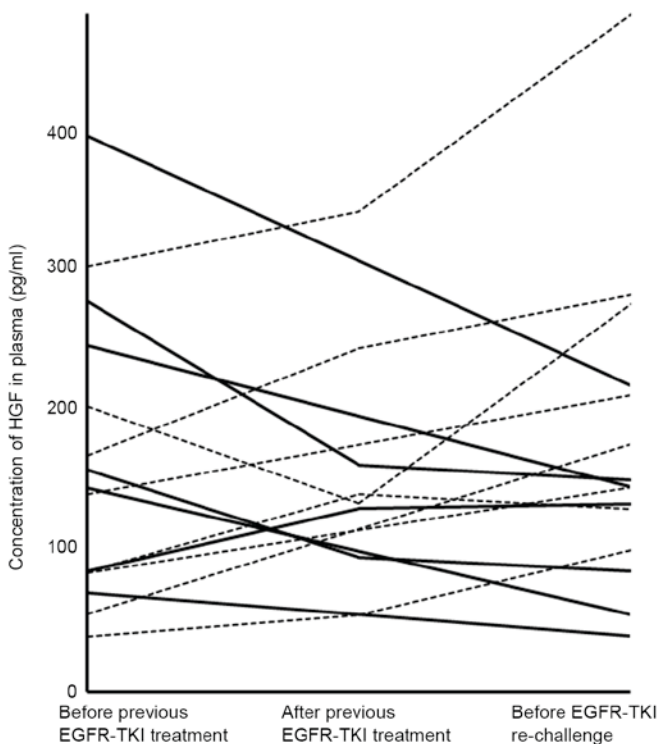


Figure 1. HGF levels in plasma during treatment. Solid lines indicate HGF levels in cases where EGFR-TKI re-challenge resulted in partial response or stable disease. Dotted lines indicate HGF levels in cases where re-challenge resulted in progressive disease. HGF, hepatocyte growth factor; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor.

However, the HGF ratio following the second re-challenge to the first re-challenge was elevated 3.2 times. As a result, the second re-challenge with gefitinib was ineffective.

Discussion

As the biological characteristics of lung cancer may alter during treatment, it is necessary to clarify the molecular events in each individual at the time of acquired resistance to EGFR-TKI and prior to re-challenge with EGFR-TKI for selection of the appropriate patient population. However, analyses of biomarkers at these times have not typically been performed due to difficulty obtaining cancer specimens during disease progression, as the majority of recurrences occur in distant sites including the brain, bone, and intrapulmonary regions (33). In addition, lung cancer is heterogeneous and the biological characteristics may vary even among metastatic lesions within a patient. Furthermore, tumor biological characteristics change frequently during treatment. Therefore, a biopsy of one lesion may not reflect the mechanisms of acquired resistance throughout the body (25,26,34-37). To solve this problem, plasma was selected as the sample for monitoring molecular events related with acquired resistance. Plasma is suitable for repeated examinations to monitor acquired resistance because collecting plasma is non-invasive, and it appears that the molecular markers detected in plasma reflect the main mechanism of acquired resistance of the entire body.

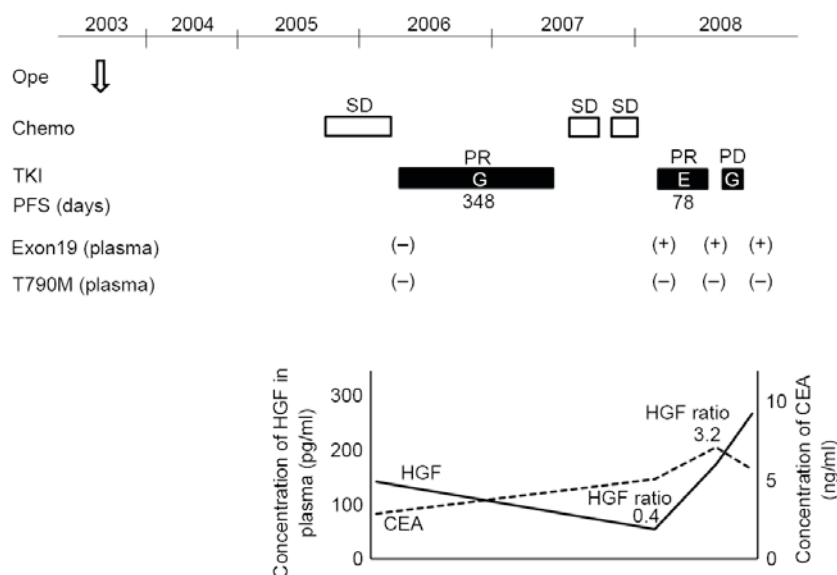


Figure 2. Combination of exon 19 deletion with T790M in plasma DNA, and HGF levels in plasma during treatment for lung adenocarcinoma. Representative results of serial analysis of the wild inhibiting PCR and quenched probe for exon 19 deletion, mutation-biased PCR and quenched probe for T790M in plasma DNA and ELISA for HGF from a lung adenocarcinoma patient is depicted. Plus and minus indicate that epidermal growth factor receptor mutation was detected or not detected, respectively. HGF, hepatocyte growth factor; CEA, carcinoembryonic antigen; PCR, polymerase chain reaction; ope, surgery; chemo, chemotherapy; TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; PR, partial response; SD, stable disease; PD, progressive disease; PFS, progression free survival; G, gefitinib; E, erlotinib.

According to results obtained using re-biopsy, *EGFR* T790M mutation and overexpression of HGF are major mechanisms of acquired resistance to EGFR-TKI. T790M was detected in 52-69% of cases and overexpression of HGF as assessed by immunohistochemistry occurs in 61% of patients who acquire resistance to EGFR-TKI (38,39). In total, 87% of patients present with either T790M or overexpression of HGF, and 26% present with the two combined (38,39). Therefore, T790M and HGF were selected to be measured in the plasma, in addition to clinical parameters, as candidate markers for predicting efficacy of re-challenge.

It has previously been reported that certain cases achieved long-term disease control for >13 months with EGFR-TKI re-challenge (40,41). Certain clinical characteristics, including response or time to progression (TTP) to previous EGFR-TKI, chemotherapy between EGFR-TKIs, and EGFR-TKI free interval have been examined as predictive markers for the success of EGFR-TKI re-challenge (16-21). The association between the occurrence of disease control (PR or SD), previous EGFR-TKI and to EGFR-TKI re-challenge has been described frequently, but other markers are controversial. Furthermore, only a small number of patients received biomarker analysis associated with acquired resistance to EGFR-TKI prior to re-treatment with EGFR-TKI.

The present study monitored *EGFR* activating mutations, T790M and HGF with plasma samples from previous EGFR-TKI treatment to re-challenge. When the transition of plasma HGF levels was compared with the effect of re-challenge, the ratio of HGF level was demonstrated to be useful as a predictive marker for the efficacy of EGFR-TKI re-challenge. As the result of comparison between acquired resistance patients and the other patients, it was concluded that a ≥ 1.5 -fold elevation of HGF was associated with acquired resistance and inefficacy of EGFR-TKI re-challenge. The

relationship between plasma biomarkers and the optimal response to EGFR-TKI re-challenge was analyzed, and the utility of using a 1.5-fold or greater elevation of HGF ratio as a negative predictive factor was demonstrated. Neither history of T790M nor elevation of HGF ratio were positive predictive factors. Considering the previous results based on re-biopsy, which revealed that mechanisms of acquired resistance including T790M and HGF overexpression may overlap, the combined systems should be reasonable. It was not possible to confirm concordance between plasma and re-biopsy. However, a previous study has reported the concordance between liquid biopsy (cell free plasma DNA and circulating tumor cell) and concurrent re-biopsy was ~60% (42). Considering this result, it is difficult to discuss the validity of liquid biopsy in comparison with re-biopsy. Therefore, the concordance between liquid biopsy and the effect of treatment is important to establish the validity of liquid biopsy. The results of the present study with T790M and HGF ratio using plasma are reflective of the clinical outcomes. These results suggested that a combination of T790M detection and HGF quantification using plasma is useful for predicting EGFR-TKI re-challenge effectiveness.

The present study had certain limitations, being a retrospective study with a small sample size. In addition, it was not possible to perform re-biopsy due to difficulty obtaining cancer specimens at the point of PD or bad general condition of patients with advanced stage cancer. Thus, it was not possible to examine associations between T790M status and HGF expression between tissue and liquid results. To demonstrate the usefulness of plasma T790M and HGF monitoring, the next step will be a prospective study with strict protocol to validate the HGF ratio in the plasma as well as T790M with plasma DNA as predictive markers for efficacy of re-challenge with EGFR-TKI. Eventually, more effective treatment strategies for

patients with NSCLC with *EGFR* activating mutation will be developed, depending on the status of T790M and HGF levels in the plasma. For example, second or the third generation *EGFR*-TKI for detection of T790M, *MET* inhibitors for the elevation of HGF levels, and *EGFR*-TKI re-challenge without detection of T790M and elevation of HGF level.

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