Usefulness of plasma HGF level for monitoring acquired resistance to EGFR tyrosine kinase inhibitors in non-small cell lung cancer

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Received June 17, 2014; Accepted September 2, 2014

DOI: 10.3892/or.2014.3560

Abstract. Monitoring of molecular markers is indispensable for deciding subsequent treatment after acquired resistance to molecular-targeted therapy. According to results using re-biopsy, *EGFR* T790M mutation and overexpression of hepatocyte growth factor (HGF) are major mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs). The aim of the present study was to assess whether quantification of HGF using peripheral blood in addition to detection of T790M with plasma DNA is useful for monitoring as an alternative to invasive re-biopsy. HGF levels in plasma were determined using ELISA and T790M mutation was detected using mutation-biased PCR and quenched probe system (MBP-QP). The median level of HGF in plasma at baseline was 140 pg/ml and was significantly higher in the advanced stage of cancer and in smokers and predicted poor survival as determined using 315 plasma samples from 225 lung cancer patients. T790M was detected with plasma DNA in 9 of 16 patients who acquired resistance to EGFR-TKIs and a greater than 1.5-fold elevation compared with pretreatment HGF levels was observed in 6 patients after acquired resistance. Eleven of 16 patients (69%) showed either HGF elevation or T790M in plasma samples, with both outcomes observed in 25% of patients; this is consistent with results based on re-biopsy reported from other laboratories. Considering these results, assessing HGF and T790M using peripheral blood could be useful for monitoring mechanisms of acquired resistance to EGFR-TKIs.

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Key words: lung cancer, HGF, T790M, plasma, monitoring system

Introduction

Clinical application of molecular targeted-therapy, such as epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), has improved the prognosis of lung cancer; the overall survival of patients with metastatic non-small cell lung cancer (NSCLC) presenting with *EGFR* mutations has risen to 27-30.5 months (1-3). Although approximately 70% of lung cancer patients with *EGFR*-activating mutations show tumor response to treatment with EGFR-TKI, they eventually acquire resistance within 10-12 months (1-3). The mechanisms of acquired resistance include *EGFR* secondary mutation, T790M, *MET* amplification, small cell transformation and overexpression of hepatocyte growth factor (HGF) (4-7). Based on this evidence, irreversible types of EGFR-TKIs and/or MET inhibitors produce marked tumor response *in vitro* and animal experiments (8-10). However, clinical trials using those agents targeted to patients who have acquired resistance to EGFR-TKIs have not been satisfactory (11-13). One of the reasons could be that biomarkers related to the mechanisms of acquired resistance were not available for these trials. Since the biological characteristics of lung cancer could be altered during treatment, it is necessary to clarify the molecular events in each individual at the time of acquired resistance to EGFR-TKIs for selection of the appropriate patient population.

We recently established a novel detection system for T790M using plasma DNA, named the mutation-biased PCR and quenched probe (MBP-QP) system, which is a sensitive, fully-automated system. Using this system, we reported that T790M was detected in plasma DNA obtained from 53% of lung cancer patients who acquired resistance to EGFR-TKIs (14). This system can be repeatedly applied to the same patients because of the non-invasiveness of collecting plasma DNA. Since most lung cancer recurrence after treatment with EGFR-TKI occurs as distant metastases, peripheral blood is appropriate for monitoring recurrence. Therefore, we chose plasma as the sample for monitoring molecular events related with acquired resistance. Since T790M and overexpression of HGF occur in 69-87% of patients who acquire resistance to EGFR-TKIs (15,16), we examined HGF and T790M using plasma samples.

HGF, a ligand for receptor tyrosine kinase, MET, contributes to the promotion of metastasis and angiogenesis (17-19). HGF is mainly secreted from fibroblasts and fat-storing cells as an inactive form, named pro-HGF, which is transformed into the active form by HGF-converting enzyme, HGF activator (HGFA), injury and glucocorticoids (18). Cancer cells such as those from lung and breast cancers have been known to be major sources of HGF, suggesting that HGF functions in both the autocrine and paracrine machinery (17-19). Neutrophils in the local environment of cancer tissue and in peripheral blood have been reported to produce *HGF* mRNA and release mature HGF (20,21). Considering these results, we assume that HGF levels in peripheral blood should reflect HGF production both in the localized environment and in the circulation throughout the whole body.

The present study therefore describes our investigation of whether quantification of the HGF level in combination with detection of T790M in peripheral blood is useful for monitoring mechanisms of acquired resistance to EGFR-TKI.

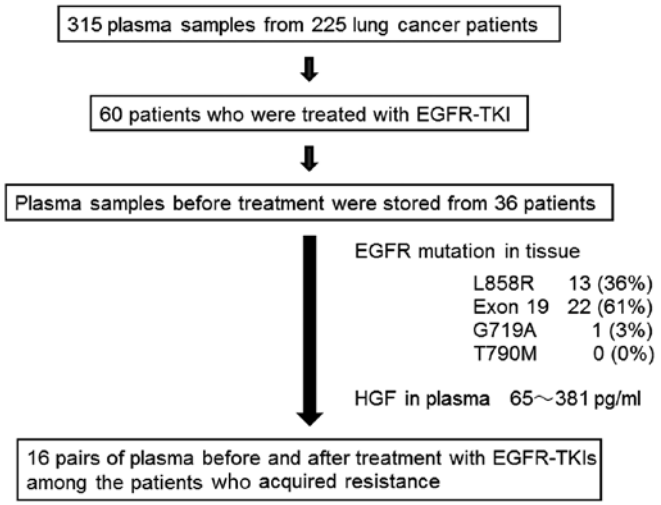

Figure 1. The schema of collecting plasma samples.

Materials and methods

Patient selection and blood samples. We obtained 315 plasma samples from 225 lung cancer patients who underwent surgical treatment or chemotherapy at Saga University Hospital from 2000 to 2013. Plasma samples were repeatedly collected from 48 patients when lung cancer progressed, from 2 to 7 times and they were obtained from 177 patients once. HGF levels in plasma were determined using ELISA as described below. Among the patients, 60 were treated with EGFR-TKIs, from whom plasma samples were randomly collected from 36 patients before treatment (Fig. 1). These samples were used for quantification of HGF as well as detection of T790M using plasma DNA. The clinical stage of the cancer was determined according to criteria in the 7th edition of the International Union Against Cancer when plasma samples were obtained (22). The criteria for acquired resistance were defined according to Jackman *et al* as follows: previous treatment with a single EGFR-TKI, a tumor that harbors an *EGFR* mutation associated with drug sensitivity or objective clinical benefit from treatment with an EGFR-TKI, as systemic progression of the disease while on continuous treatment with an EGFR-TKI within the last 30 days and no intervening systemic therapy between cessation of EGFR-TKI and initiation of new therapy (23). The study protocol was approved by the Clinical Research Ethics Committee of Saga University. All patients provided informed consent for obtaining blood according to the Declaration of Helsinki.

Quantification of the HGF level in plasma. Peripheral blood samples from lung cancer patients were collected into tubes containing 3.8% citric acid. Plasma was immediately separated from blood cells by 3,000 rpm centrifugation at 4˚C for 20 min. Supernatants were collected and stored at -80˚C until assays were performed. The HGF level in plasma was measured by enzyme-linked immunosorbent assay (Immunis HGF EIA; B-Bridge International, Mountain View, CA, USA; limit of detection, 100 pg/ml), according to the manufacturer's recommendations. Fifty microliters of 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. HGF concentrations were determined by comparison with standard curves.

DNA extraction from plasma and detection of the EGFR T790M mutation. DNA was isolated from 200 μl of patient plasma using a QIAamp® DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. T790M mutation was detected using the MBP-QP method as described previously (14). Briefly, MBP-QP is a fully automated system with two steps: mutation-biased PCR (MBP) and quenched probe (QP) system using i-densy (ARKRAY Inc., Kyoto, Japan). For MBP, the primers for the wild-type and mutant-type were mixed with genomic DNA, which leads to high specificity since each primer could be competitively hybridized to the wild-type and mutant sequences. In addition, the length of the reverse primer for the mutant was longer than that for the wild-type and the annealing temperature was designed to be optimum to the mutant primer, resulting in higher efficiency of amplification of the mutant sequence. The presence of T790M in the amplified sequences was determined by monitoring the fluorescence intensity of a TAMRA-conjugated, guanine-specific quench fluorophore probe (QProbe; J-Bio21, Tokyo, Japan), which is complementary to T790M. The dissociation temperatures were 66˚C for the mutant and 59˚C for the wild-type.

Statistical analysis. The association between HGF levels and clinicopathological characteristics was tested using the nonparametric Mann-Whitney U test for continuous variables and Kruskal-Wallis analysis was used for assessing whether the distribution of HGF differed among the pathological stages. Survival rate was calculated according to the Kaplan-Meier method with differences assessed using the log rank test. Cox proportional hazards regression analysis, with adjustment for potentially confounding variables (gender, smoking status, histology, pathological stage and EGR mutations), was used to calculate the hazard ratio (HR) and 95% confidence internal (CI) of survival outcome of lung cancer patients. All statistical analyses were conducted using IBM SPSS Statistics 19 (SPSS Inc., IBM Company).

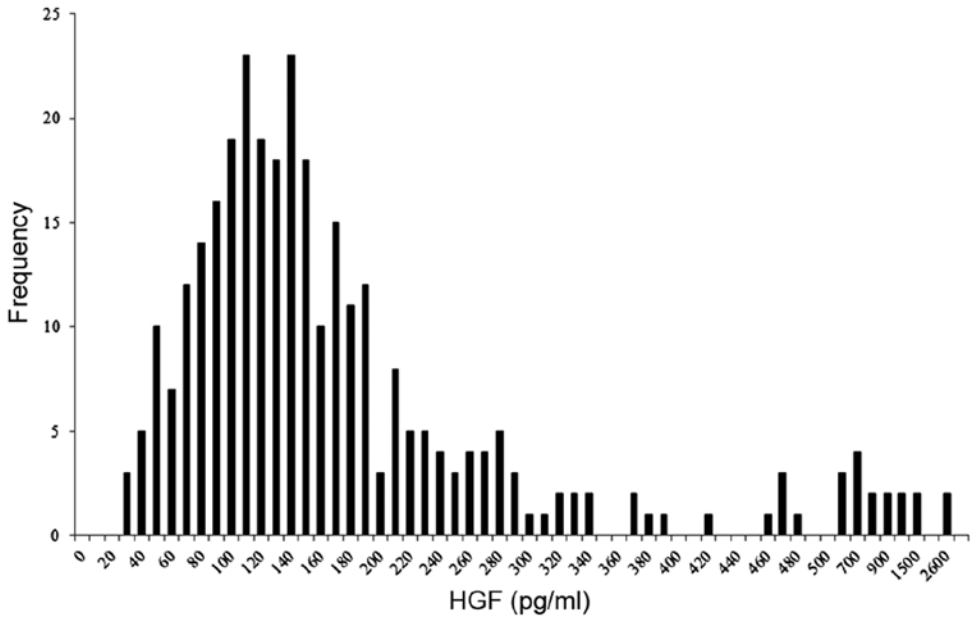

Figure 2. Distribution of hepatocyte growth factor (HGF) level in plasma obtained from lung cancer patients. HGF was quantified using ELISA. Median plasma HGF level was 140 pg/ml.

Table I. Characteristics of the study patients.

Characteristics	Data, n (%)
Total	225
Age (years)	
Median	68
Range	41-88
Gender	
Male	147 (65)
Female	78 (35)
Smoking	
Smoker	146 (65)
Non-smoker	79 (35)
Histology	
Squamous cell carcinoma	39 (17)
Adenocarcinoma	147 (65)
Small cell carcinoma	21 (9)
Others	18 (9)
Pathological stage	
I	79 (35)
II	20 (9)
III	62 (28)
IV	64 (28)
EGFR mutation	
L858R	31 (14)
Exon 19 deletion	44 (19)
Others	7 (3)
Negative	78 (35)
Unknown	65 (29)

EGFR, epidermal growth factor receptor.

Results

Clinicopathological characteristics of the lung cancer patients with high HGF levels in plasma. The 225 lung cancer patients comprised 91% non-small cell and 9% small cell lung cancer cases (Table I). Sixty-five percent were adenocarcinoma cases and 36% expressed *EGFR* mutations (L858R, exon 19 deletions, or others). The lower limit of HGF quantification was 100 pg/ml; 31 of the 315 plasma samples had HGF levels below that limit. According to calculation by a standard curve, the distribution of HGF levels is shown in Fig. 2. The median HGF level was 140 pg/ml and the upper end of the range was 2,600 pg/ml. The correlation with clinicopathological characteristics showed that the HGF levels were significantly higher among patients with advanced stage and among smokers (Fig. 3A and B). Associations between HGF levels and either histological type (Fig. 3C) or gender (Fig. 3D) were not observed. Prognosis according to HGF level in plasma, comparing HGF greater than the median and HGF less than or equal to the median, is shown in Fig. 4. The median survival time with high HGF was 409 days whereas that with low HGF was not achieved, so that the high HGF group had significantly shorter survival (log rank $p<0.001$). In addition to the HGF level in plasma, possible prognostic factors, including gender, age, pathological stage, histology, smoking status and *EGFR* mutation, were analyzed. Based on a multivariate Cox proportional hazards model, Table II shows that pathological stage, *EGFR* mutation status and HGF level in plasma had significant effects on survival even when simultaneously adjusted. *EGFR* mutation-positive cases showed favorable survival, whereas HGF level in plasma, as well as pathological stage, was a predictor of poor survival. These results suggest that clinicopathological characteristics of the patients with high HGF levels in plasma are equivalent to those of patients with high HGF in lung cancer tissues as previously reported in other laboratories (24,25).

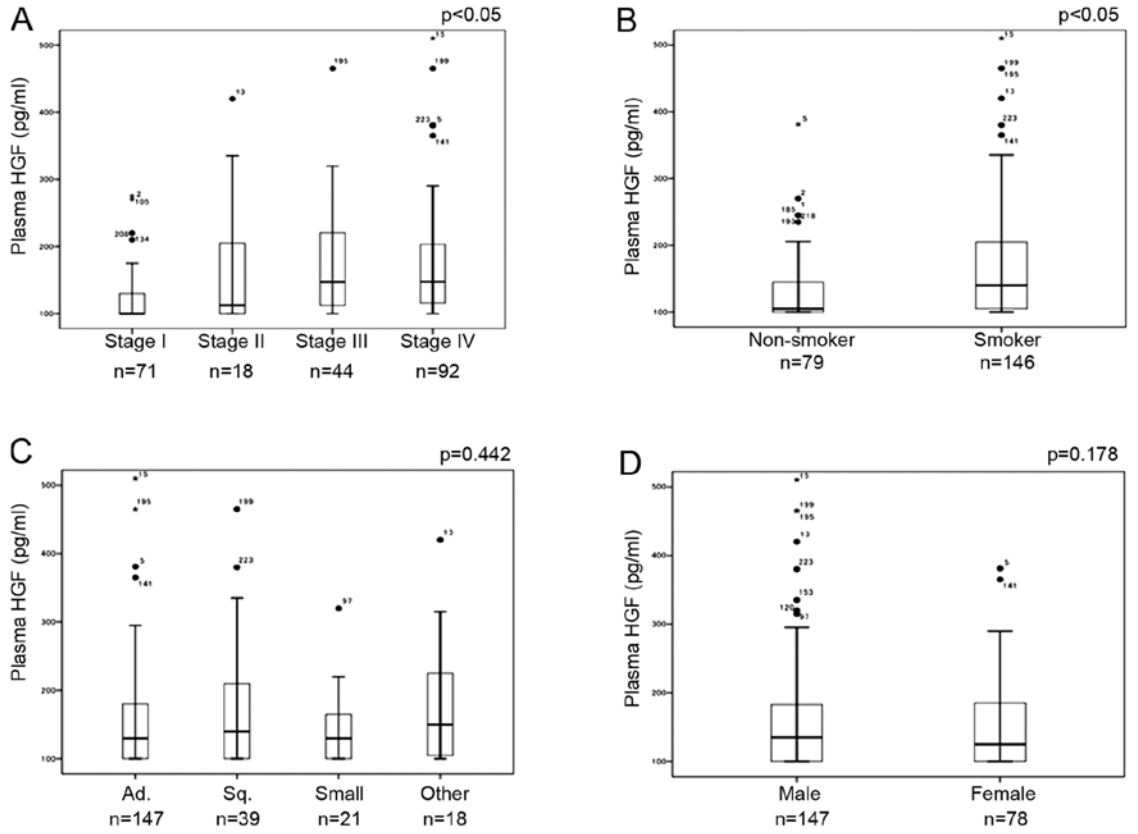

Figure 3. Distribution of hepatocyte growth factor (HGF) level in plasma obtained from lung cancer patients according to clinical stage (A), smoking status (B), histology (C), and gender (D).

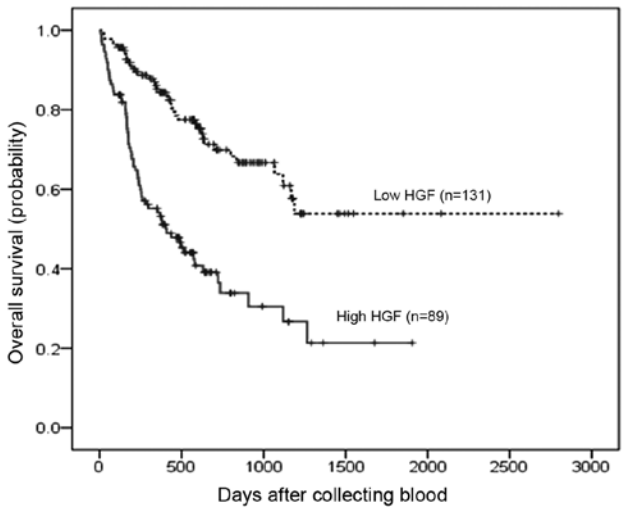

Figure 4. Cumulative Kaplan-Meier survival curves stratified according to hepatocyte growth factor (HGF) level in plasma among all patients. High and low HGF was defined as higher than, or less than or equal to, the median HGF level, 140 pg/ml, respectively.

Table II. Survival outcome by multivariate Cox proportional hazards analysis for the lung cancer patients.

Factors	HR (95% CI)	P-value
Age	1.01 (0.99-1.03)	0.51
Gender (male vs. female)	0.89 (0.43-1.82)	0.74
Smoking status (non-smoker vs. smoker)	0.66 (0.31-1.39)	0.27
Histology (Ad vs. others)	1.14 (0.69-1.90)	0.61
Pathological stage (IV vs. I, II, III)	5.70 (3.63-8.95)	<0.001
EGFR mutation (negative, unknown vs positive)	2.20 (1.23-3.93)	0.008
HGF (high vs. low)	2.52 (1.67-3.80)	<0.001

HR, hazard ratio; CI, confidence interval; EGFR, epidermal growth factor receptor; HGF, hepatocyte growth factor.

Elevation of HGF level and/or T790M using plasma samples were frequently observed among the patients who acquired resistance to EGFR-TKIs. Among the 225 patients examined in this study, 60 were treated with EGFR-TKIs, and plasma samples were randomly collected from 36 patients before treatment (Fig. 1). *EGFR* mutations including L858R in 13 patients and exon 19 deletions in 22 patients were observed in lung cancer tissues obtained from 36 patients before treatment with EGFR-TKIs. T790M was not detected in cancer tissues in any patients. The range of HGF levels in plasma was from 65 to 381 pg/ml before treatment. Among the patients who

Table III. Plasma HGF levels and T790M in plasma DNA from the lung cancer patients who acquired resistance to EGFR-TKIs.

	HGF (pg/ml)			T790M	
	Before	After	Ratio	Before	After
1	160	200	1.3	Negative	Negative
2	381	510	1.3	Negative	Positive
3	170	1235	7.3	Negative	Positive
4	130	166	1.3	Negative	Positive
5	120	340	2.8	Negative	Positive
6	680	725	1.1	Negative	Positive
7	175	270	1.5	Negative	Negative
8	245	145	0.59	Negative	Negative
9	145	325	2.2	Negative	Negative
10	90	140	1.6	Negative	Positive
11	301	157	0.52	Negative	Positive
12	277	160	0.58	Negative	Negative
13	130	265	2.0	Negative	Positive
14	105	79	0.76	Negative	Negative
15	185	190	1.0	Negative	Negative
16	202	240	1.2	Negative	Positive

HGF, hepatocyte growth factor; EGFR-TKIs, epidermal growth factor receptor tyrosine kinase inhibitors.

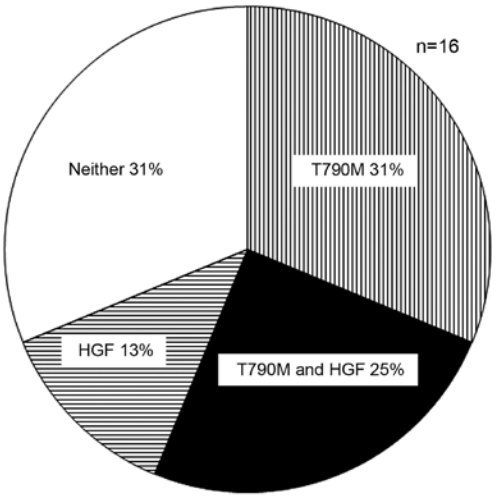

Figure 5. Frequency of hepatocyte growth factor (HGF) level elevation in plasma and *EGFR* T790M mutation detection with circulating plasma DNA among the patients who acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs). Elevation of HGF in plasma was defined as the ratio of the HGF level after acquired resistance to that before treatment equal to at least 1.5.

acquired resistance to EGFR-TKIs, 16 pairs of plasma before treatment with EGFR-TKIs and after acquired resistance were obtained (Table III). Since the clinicopathological characteristics with high HGF level in plasma were similar to that in lung cancer tissues, it is possible that HGF in plasma would reflect the local HGF level in cancer tissue. Therefore, we next investigated whether HGF levels were elevated at the time point of acquired resistance to EGFR-TKIs compared to those before treatment. *EGFR* T790M mutation with plasma DNA was also examined. Plasma HGF levels ranged from 90 to 680 and 79 to 1,235 pg/ml before treatment of EGFR-TKI and after acquired resistance, respectively. The ratio of HGF level after acquired resistance to that before treatment ranged from 0.52 to 7.3 and 6 patients showed a >1.5-fold elevation in the HGF. T790M was detected with plasma DNA in 9 patients after acquired resistance to EGFR-TKIs. Eleven of the 16 patients (69%) showed either an HGF elevation (≤1.5-fold) or T790M with plasma samples and elevations in both were observed in 4 patients (25%) (Fig. 5).

Discussion

HGF plays a central role in cancer progression, including proliferation of cancer cells, invasion, angiogenesis and metastasis (17-19). From the viewpoint of these biological activities, HGF has been investigated as a candidate prognostic marker for various cancers including colon, stomach, prostate and multiple myeloma (24-27). In lung cancer, HGF overexpression has been reported to be a prognostic factor using tissue samples (28,29). As an alternative to using cancer tissue specimens, we showed that the HGF levels in plasma were higher in advanced stage and among smokers and it is a prognostic marker for lung cancer, which is independent of other clinicopathological factors.

The mechanisms of acquired resistance to EGFR-TKIs have been reported to be *EGFR* secondary mutation, T790M, *MET* or *HER2* amplification and small cell transformation (4-6,15,16). In addition to those mechanisms, HGF induced gefitinib, EGFR-TKI, resistance of lung adenocarcinoma cell lines carrying *EGFR* activating mutations and HGF overexpression was observed in cancer tissues from patients who acquired resistance to EGFR-TKIs (7). According to the results using re-biopsy specimens in other laboratories, T790M was detected in 51-69% and HGF overexpression examined by immunohistochemistry was observed in 61% of cases (15,16,30). Although re-biopsy would be the most reliable method for determination of the mechanisms of acquired resistance to EGFR-TKIs, it is associated with various issues. One is that re-biopsy is invasive, since most lung cancer recurrence occurs as distant metastases in liver, bone, brain, adrenal gland and intrapulmonary regions. The other is that the molecular characteristics sometimes vary among the metastatic lesions, suggesting that a biopsy specimen in one lesion would not reflect the entire body (31). Considering these issues, peripheral blood could be an appropriate sample for determining the dominant molecular alterations in the entire body. Since collecting peripheral blood is non-invasive, it is suitable for monitoring acquired resistance, which requires repeated examinations. We showed that T790M was detected in 56% of cases and elevation of HGF was observed in 38% of cases. Although the frequency of HGF elevation was lower than that using re-biopsy, the T790M detection rate was equivalent. The possible reason for the lower frequency of HGF elevation in plasma could be that cells expressing HGF exist not only in cancer tissue but also in peripheral blood, resulting in a high background level of HGF in plasma.

These mechanisms of acquired resistance sometimes co-exist such as T790M and *MET* amplification, small cell transformation and T790M and HGF overexpression and T790M (15,16). This phenomenon may cause primary resistance to second generation EGFR-TKIs and therefore it is critical for making decisions whether combination therapy is needed or not. Our results revealed that co-existence of T790M and HGF elevation was observed in 25% (4/16) of the patients who acquired resistance to EGFR-TKIs, which is also equivalent to that using re-biopsy. Combining detection systems for HGF and T790M using plasma samples enabled us to detect the mechanisms of acquired resistance to EGFR-TKIs in 69% of patients who acquired resistance to EGFR-TKIs. A prospective study to investigate the utility of these detection systems for predicting the anticancer effects of next generation EGFR-TKIs and/or MET inhibitors is worthy of investigation.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Young Scientists (B) 25870518. The authors are grateful to Dr John B. Cologne for suggestions regarding the statistical analysis.

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