Current Status and Problems of T790M Detection, a Molecular Biomarker of Acquired Resistance to EGFR Tyrosine Kinase Inhibitors, with Liquid Biopsy and Re-biopsy

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Abstract. *Background/Aim:* The purpose of this study was to consider appropriate application of liquid and re-biopsy through analysis of current status in practice. *Patients and Methods:* We performed a retrospective analysis of 22 patients with epidermal growth factor receptor (EGFR) mutation-positive non-small cell lung cancer who exhibited 1st/2nd generation EGFR-tyrosine kinase inhibitors resistance. The cobas® method was used to detect T790M with re-biopsy and the mutation-biased PCR and quenched probe method was used with liquid biopsy. *Results:* T790M detection rate was 52% with re-biopsy and 58% with liquid biopsy. The concordance between tissue and plasma was 58%. One patient who was T790M-positive with liquid biopsy showed heterogeneity among metastatic lesions in terms of osimertinib efficacy, as revealed by T790M detection with re-biopsy. *Conclusion:* Liquid biopsy reflects the whole body, whereas re-biopsy is useful for spatial diagnosis. Considering these characteristics, a combination of liquid and re-biopsy contribute to enhanced treatment.

Treatment outcome in patients with non-small cell lung cancer patients has substantially improved with the development of molecular targeted therapeutic agents, such as epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) (1, 2), anaplastic lymphoma kinase (ALK)

inhibitor (3, 4), and ROS 1 inhibitor (5). However, acquired resistance occurs after about one year in most cases, so that elucidating and overcoming the resistance mechanism is an important goal. Liquid biopsy using circulating tumor DNA (ctDNA) has come into use throughout the world for this purpose, and it was recently approved for clinical use in Japan following its approval in the United State and Europe.

T790M, a gate keeper mutation of *EGFR*, is found in about 50-60% of cases that are resistant to EGFR-TKI (6, 7), and examination using the cobas® EGFR Mutation Test v2 (Roche Molecular Systems, Inc., CA, USA), the companion diagnostic for osimertinib, is mandatory for treatment with osimertinib, a third generation EGFR-TKI targeting T790M. At first, re-biopsy using tissue samples was necessary for this examination, but thereafter liquid biopsy using ctDNA was approved. However, it remains controversial whether liquid biopsy is effective for predicting osimertinib efficacy. In the AURA Study, overall response rate was similar among patients who were T790M-positive with either tissue or ctDNA. However, 35-46% of patients who were T790M negative with ctDNA nevertheless responded to osimertinib, suggesting that liquid biopsy generates a greater number of false-negatives than does re-biopsy (8, 9). On the contrary, among patients who were T790M-negative in re-biopsy, the T790M detection rate with liquid biopsy was over 30% using a highly sensitive mutation detection system, BEAMing (8). The allelic frequency of T790M was less than 10%, and osimertinib efficacy was limited in those patients. Concordance between liquid biopsy and re-biopsy in the detection of T790M was approximately 70%, whereas that with regard to activating mutations—such as L858R and exon 19 deletions—was more than 90% (8); the difference is assumed to be due to increased tumor heterogeneity after acquired resistance. These results suggest that liquid biopsy for detecting mutations related to acquired resistance needs

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Key Words: T790M, liquid biopsy, re-biopsy, non-small cell lung cancer.

to be firmly established for accurately predicting the efficacy of molecular targeted therapy. Based on these findings, the International Association for the Study of Lung Cancer (IASLC) consensus statement and the Japan Lung Cancer Society recommended that tissue biopsy be given priority and plasma testing be considered only in difficult cases (10). Furthermore, the ESMO Clinical Practice Guidelines state that plasma DNA specimens should be considered as a surrogate for tissue DNA specimens (11).

We examined T790M with liquid biopsy using the mutation-biased PCR and quenched probe (MBP-QP) method, and we have shown that T790M was positive in 58% of patients who acquired resistance to EGFR-TKI in a retrospective study and 40% in a multi-institutional prospective study (12). Using this system, we examined T790M with liquid biopsy concomitant with re-biopsy in patients with non-small cell lung cancer who were treated with EGFR-TKI in practice. In this article, we report the results of our retrospective analysis of T790M to compare liquid biopsy and re-biopsy, and we discuss how these systems may be used appropriately together.

Patients and Methods

Patients. We performed a retrospective analysis of 22 patients with *EGFR* mutation-positive non-small cell lung cancer (NSCLC) who exhibited 1st or 2nd generation EGFR-TKI resistance. The patients were treated at the Saga University Medical School Hospital from April 2016 to June 2017. Osimertinib was approved in Japan on April 2016, and thereafter we started performing re-biopsy to determine indications for its use. All patients underwent re-biopsy; plasma specimens were taken at the same time as re-biopsy, with 24 samples obtained from 21 patients. The study protocol was approved by the Saga University Clinical Research Ethics Committee, and all patients gave informed consent for the use of tissue and plasma specimens.

***EGFR* gene mutation test.** The cobas® *EGFR* Mutation Test v2 (Roche Molecular Diagnostics, Pleasanton, CA, USA) and the MBP-QP method were used to assess *EGFR* gene mutation. The former, a real-time PCR method using Taqman probe, is a companion diagnostic for osimertinib and was performed by SRL Inc. (Tokyo, Japan) under coverage by medical insurance. The MBP-QP method is a fully automated *EGFR* mutation detection system developed in our laboratory (13, 14). The measurement principle can be described briefly as follows. The method consists of two steps: an MBP method with improved PCR and a QP method for identifying mutated fragments using a quenching probe. In the MBP process, PCR is performed by mixing complementary primers of wild-type and mutant type. By altering the length of the antisense primer and matching it to the mutation condition, amplification efficiency of the mutant fragment is improved. In the QP process, because the quenching probe is completely matched to the mutant type, it is partially incompatible with the wild type; as a result, the dissociation temperature differs and so it is possible to distinguish between wild type and mutant type. The fully-automatic assessment is completed in one-and-a-half hours using i-densy™ IS-5320

Table I. Patient characteristics.

	n=22
Age (years)	66 (43-87)
Gender	
Female	11
Male	11
Smoking index	
Never smoker	13
Ex-smoker	9
Mutation status	
Del 19	10
L858R	12
Histology	
Adenocarcinoma	22
Other	0
TKI treatment history before re-biopsy	
Gefitinib	8
Gefitinib→Erlotinib	2
Gefitinib→Afatinib	5
Gefitinib→Erlotinib→Afatinib	2
Afatinib	3
Afatinib→Gefitinib	2

(ARKRAY, Inc., Kyoto, Japan) for detection. For detection of *EGFR* exon 19 deletions, the wild inhibiting PCR and quenched probe system (WIP-QP) was used, as described previously (14). With tissues or cytological specimens, activating and T790M mutations were measured using cobas v2.0. With plasma specimens, these mutations were measured with MBP-QP for T790M and L858R, and with WIP-QP for exon 19 deletions. T790M mutations in tissue specimens from some patients were also measured with the MBP-QP method. After the “EGFR T790M plasma test result support program” by AstraZeneca was started, plasma T790M was examined in some patients using cobas v2.0.

Statistical analysis. Tumor response was evaluated according to the Response Evaluation Criteria In Solid Tumors (RECIST) version 1.1. Differences in T790M mutation detection rates according to type of gene mutation were tested with the chi-square test.

Results

Clinical background and details of re-biopsy. From April 2016 to June 2017, all 22 patients who had become resistant to the 1st and 2nd generation EGFR-TKI underwent re-biopsy. The median age was 66 years (range=43-87 years). By *EGFR* mutation type, there were 10 patients with del 19 and 12 patients with L858R. All patients were diagnosed with adenocarcinoma. The distribution of treatment with EGFR-TKI before re-biopsy was 8 patients with gefitinib alone, 2 with gefitinib followed by erlotinib, 5 with gefitinib followed by afatinib, 2 with gefitinib followed by erlotinib and then afatinib, 3 with afatinib alone, and 2 with afatinib followed by gefitinib (Table I). Table II shows details of re-

Table II. Tumor sites and methods of re-biopsy.

Patient	Mutation status	Site of re-biopsy	Sampling method	Time to re-biopsy from IC (days)	Tissue or cytology (cobas v2.0)			Liquid (MBP-QP)	
					Detection of cancer cell	Detection of activating mutation	Detection of T790M	Detection of activating mutation	Detection of T790M
1	Del19	Bone	Surgery	33	Y	Y	N	Y	Y
		Liver	NUG	2	Y	Y	Y	Y	Y
2	Del19	Primary	TBLB	21	N	NE	NE	NA	NA
		Primary	NCG	4	Y	Y	N	N	N
3	Del19	PM	Surgery	17	Y	Y	Y	N	N
4	L858R	Bone	NCG	38	Y	Y	Y	Y	Y
5	Del19	Primary	TBLB	10	Y	Y	N	N	Y
6	L858R	Pleura	NCG	5	Y	Y	Y	NE	Y
7	L858R	LN	Surgery	11	Y	Y	N	Y	N
		LN	Surgery	14	Y	Y	N	Y	Y
		Liver	NUG	8	Y	Y	N	Y	Y
8	L858R	Primary	Surgery	14	Y	Y	Y	Y	Y
9	Del19	Bone	Surgery	39	Y	N	N	N	Y
10	L858R	LN	Surgery	28	Y	Y	Y	Y	Y
11	Del19	Pleura	NCG	2	Y	Y	Y	Y	N
12	L858R	Liver	NUG	13	Y	Y	Y	NA	NA
13	L858R	Bone	NCG	7	Y	N	N	Y	N
14	L858R	Bone	NCG	9	Y	Y	N	Y	Y
15	L858R	Bone	NCG	6	Y	Y	Y	Y	Y
16	L858R	Pleura	NCG	8	Y	Y	N	N	N
17	Del19	Pleural effusion	Lavage	1	Y	Y	Y	N	Y
18	Del19	Bone	NCG	12	Y	Y	Y	Y	Y
19	Del19	Primary	TBLB	18	Y	Y	Y	N	N
20	Del19	PM	TBLB	26	Y	Y	Y	Y	N
21	L858R	Primary	TBLB	25	Y	Y	N	N	N
22	L858R	Primary	NCG	16	Y	Y	N	N	N

PM: Pulmonary metastases; LN: lymph node; NUG: needle under ultrasonic guidance; TBLB: transbronchial lung biopsy; NCG: needle under CT guidance; NE: not evaluated; IC: informed consent; NA: not available.

biopsy in each case. Re-biopsy site was most frequently in bone and primary sites (7 samples each), and less frequently in liver, pleura, lymph node (3 each), intrapulmonary metastases (2), and pleural effusion (1). As re-biopsy procedures, computed tomography (CT)-guided biopsies were most frequently performed (10 samples), followed by surgical biopsies (7), transbronchial lung biopsies (5), ultrasound-guided biopsies (3), and pleural effusion puncture (1). One case of atypical angina occurred as a complication of transbronchial lung biopsy, but the patient recovered completely. The median interval from explanation and patient consent to re-biopsy was 12.5 days (range: 1-39). The detection rate of cancer cells in re-biopsy (success rate) was 96%. In re-biopsy, activating mutations by cobas were observed in all samples except those from patients 9 and 13. The detection rate of T790M mutation with re-biopsy was 52% (13/25) in all samples: 64% (7/11) in cases with del 19 and 43% (6/14) in cases with L858R. (Table III). The T790M detection rate was also examined with the MBP-QP method

in plasma samples collected at the same time: the detection rate was 58% (14/24) in all samples, 55% (6/11) in cases with del 19, and 62% (8/13) in cases with L858R. The concordance rate of T790M between tissue and cell specimens (assessed with cobas) and plasma specimens (assessed with MBP-QP) in terms of rate of T790M was 58% (14/24). The detection rate of the activation mutations (exon 19 deletions or L858R) in plasma specimens by MBP-QP was 64% (14/22), and the concordance between tissue and plasma samples was 64% (14/22).

Correlation between T790M status and response to osimertinib. The therapeutic effect in 14 patients who received osimertinib was studied retrospectively (Table IV). Eight patients who were positive for T790M in both tissue and plasma specimens were all partial response, except for one case, who had no evaluable lesions. Four patients (two partial response and two stable disease) who were positive for T790M in tissue specimens but not plasma samples

Table III. *EGFR* mutation detection rate and concordance between re-biopsy and liquid biopsy.

	Detection rate of T790M	
Tissue or cytology (cobas v2) (n=25)	13/25 (52%)	
Del 19	7/11 (64%)	<i>p</i> =0.43
L858R	6/14 (43%)	
Liquid (MBP-QP) (n=24)	14/24 (58%)	
Del 19	6/11 (55%)	<i>p</i> =0.73
L858R	8/13 (62%)	
Concordance of T790M detection (cobas v2.0)	Tissue or cytology	
	+	−
Liquid (MBP-QP)		
+	8	6
−	4	6
Concordance of activating mutation (cobas v2.0)	Tissue or cytology	
	+	−
Liquid (MBP-QP)		
+	13	1
−	7	1

Table IV. Correlation between T790M status and response to osimertinib.

Patient	Tissue or cytology (cobas v2.0)	Liquid (MBP-QP)	Response
1	N (bone)	Y	PR
	Y (liver)	Y	
4	Y	Y	PR
6	Y	Y	PR
8	Y	Y	PR
10	Y	Y	PR
15	Y	Y	NE
17	Y	Y	PR
18	Y	Y	PR
3	Y	N	SD
11	Y	N	SD
19	Y	N	PR
20	Y	N	PR
7	N (LNs)	Y	PD
	N (liver)*	Y	
14	N	Y	NE

LN: Lymph node; PR: partial response; NE: not evaluated; SD: stable disease; PD: progressive disease. *T790M was detected by the MBP-QP method.

showed positive therapeutic effects. There were two patients in whom T790M was positive only in plasma specimens; treatment efficacy was progressive disease (PD) in one of these patients, and not evaluable in the other. As for the patient with PD (patient 7), re-biopsy was performed twice on axillary lymph nodes and subsequently on liver. Response to osimertinib was mixed based on T790M with two re-biopsies, resulting in evaluation as PD. Another patient who was T790M positive only with liquid biopsy (patient 14) showed declining levels of tumor marker, but treatment efficacy was not evaluated because of an exacerbated condition.

Two patients (patients 1 and 7), who underwent re-biopsy twice or three times, are now described in detail. Patient 1 is a 43-year-old non-smoker with *EGFR* del 19 and clinical stage cT1aN2M1b (UICC-7, multiple brain and bone metastases; Figure 1). Afatinib was administered as the first line therapy after palliative irradiation for bone metastasis. Plasma T790M measured by MBP-QP was negative before and during afatinib administration, but turned positive after PD. Because osimertinib was not marketed at that time, we chose cytotoxic agents (carboplatin and pemetrexed plus bevacizumab) as the second line therapy, but exacerbation with multiple bone metastases was observed. Therefore, re-biopsy was performed from the left ileum; T790M was negative by the cobas method but positive by MBP-QP. In plasma collected at the same time, T790M was also positive by MBP-QP, but because T790M

was negative by the cobas method, osimertinib could not be administered. The patient did not respond to re-administration of afatinib and showed disturbance of consciousness due to exacerbation of meningeal dissemination. An additional re-biopsy was performed from a liver metastasis; T790M was positive by both cobas and MBP-QP, leading to administration of osimertinib. Thereafter, disturbance of consciousness and general condition improved, and the patient is still being seen as an outpatient.

Another patient, patient 7, is a 74-year-old man with smoking index 1575. The clinical stage was cT2bN1M1b (UICC-7, liver, bone, axillary lymph node, and muscle), and *EGFR* L858R was positive (Figure 2A). Gefitinib was administered as the first line therapy, and a combination of gefitinib and pemetrexed was administered in a clinical trial as the second line therapy. Plasma T790M was positive by MBP-QP at PD during the second line therapy. Because osimertinib was not marketed at that time, afatinib was started as the third-line therapy and the patient responded only temporarily. Plasma T790M was repeatedly positive by MBP-QP. After PD to chemotherapy, re-biopsy from the axillary lymph node metastasis was performed, and T790M was negative by cobas but positive by MBP-QP. Administering a single agent, *nab*-paclitaxel, as the fifth line also produced no effect; T790M status in a second re-biopsy from the re-enlarged axillary lymph node metastasis was the same as that of the first re-biopsy and so osimertinib still

Figure 1. Treatment course and T790M status of patient 1. The black arrow indicates a bone biopsy site, and the white arrow indicates a liver biopsy site.

could not be administered. A third re-biopsy was performed from liver metastasis, but both cobas and MBP-QP produced negative results. In December 2016, AstraZeneca launched the “EGFR T790M plasma test result support program” as an interim measure until insurance approval, and T790M proved positive so that finally osimertinib could be administered. The effect of osimertinib is shown in Figure 2B. The axillary lymph node metastases—which were T790M positive only by MBP-QP—were markedly reduced, but the liver metastases—which were negative for T790M in both cobas and MBP-QP—showed no effect.

Discussion

In this study, we retrospectively analyzed T790M mutation with liquid biopsy and compared the results to re-biopsy in actual practice. Re-biopsy was performed in all patients who acquired resistance to 1st or 2nd generation EGFR-TKI, and the rate of detection of cancer cells in re-biopsy specimens was 96%. T790M detection rate in tissue or cytological specimens by cobas v2.0 and in ctDNA by the MBP-QP method were 52% and 58%, respectively. Concordance between liquid biopsy and re-biopsy was 58%. Osimertinib

was clinically effective among patients who were T790M-positive with both liquid biopsy and re-biopsy, but its efficacy was mixed in patients who were T790M-positive with only liquid biopsy.

Although an initial diagnosis of lung cancer is usually made with less invasive procedures using bronchoscopy, the proportion of distant metastases increases after acquired resistance to treatment, and invasive re-biopsy is often required. In this study, extrathoracic metastases were observed in 50% of patients, and 27% required surgical resection. Alcila *et al.* reported that surgery—such as lung resection, thoracoscopic lung biopsy, brain metastasis resection, laminectomy, and adrenalectomy—accounted for 40% of re-biopsies (15). In a retrospective analysis of re-biopsy in Japan, it was reported that the occurrence of re-biopsy by surgical excision exceeds that of initial biopsy (7.8% vs. 1.8%) (16). Although most cases required invasive procedures for re-biopsy, median time to re-biopsy from obtaining informed consent was 12.5 days, indicating that cooperation with the surgical or radiological department is indispensable. Complications occurred in one patient who suffered from variant angina during bronchoscopy, but he recovered promptly. It was also reported that in the retrospective analysis

Figure 2. A. Treatment course and T790M status of patient 7. After the “EGFR T790M plasma test result support program” by AstraZeneca was started, cobas v2.0 test using a plasma specimen was performed. B. The contrast CT images of patient 7 are shown. After administration of osimertinib, right axillary lymph node metastases shrank (a, red circle), and multiple liver metastases became enlarged (b).

of 111 histological re-biopsies, it can be highly successful and feasible by optimal procedural selection (17). In short, re-biopsy is reasonably feasible in practice.

The concordance between tissue and ctDNA values of T790M was 58%, which is similar to that in other reports (12, 18-20). One explanation for the lack of high concordance is inter- and intra-tumor heterogeneity. Compared to *EGFR* activating mutations, concordance between liquid biopsy and re-biopsy values of T790M is relatively low. Tumor heterogeneity increases during the process of cancer evolution that occurs with treatment modification and subsequent repeated acquired resistance (21-23). In addition, there is a report that the expression of programmed death-ligand 1 (PD-L1) changes after nivolumab and chemotherapy treatment, and it is considered that the tumor environment constantly changes due to modification by treatment (24). At the time of diagnosis, *EGFR* activated mutations are uniform, but genetic alterations related to acquired resistance result in heterogeneity, leading to differences in detection rate. Another explanation for inconsistency is different progression status at the time when ctDNA is obtained. According to our previous prospective study, T790M was observed less frequently in patients with PD whose diseases were limited to the thorax than in patients with the appearance of new lesions, which were mostly distant metastases (12). In our study, four patients with T790M detected only by re-biopsy had metastatic lesions limited to the thoracic cage.

Most patients who are T790M-positive received benefit from osimertinib, but the efficacy differed according to whether T790M was detected by liquid biopsy or re-biopsy. All patients who were T790M-positive in both liquid biopsy and re-biopsy showed a partial response, except for one patient whose target lesions were absent but tumor marker was declining after treatment with osimertinib. Four patients with T790M detected only by re-biopsy also responded to osimertinib. However, the effect of osimertinib on metastatic lesions in patient 7 differed between the axillary lymph node and the liver; the former lesion shrank but the latter lesion became enlarged, which is consistent with T790M being positive in the axillary lymph node and negative in the hepatic metastatic lesion detected by MBP-QP. Such a difference in detection sensitivity may be the cause of the fact that, overall, the MBP-QP method detects T790M with a sensitivity of less than 0.3% whereas cobas v2.0 detects it with that of 3-5% (13, 25).

There exist several limitations in this paper. First, since it is a single center retrospective study, the number of samples was not sufficient. Also, the current standard method of plasma T790M detection is the cobas® *EGFR* Mutation Test v2 only. For that purpose, it is necessary to investigate whether the MBP-QP method is useful as a method of plasma T790M detection and investigate the correlation between the results of the MBP-QP method and the response to osimertinib. We have now performed a multicenter prospective study for these purposes,

In summary, liquid biopsy reflects the whole body and enables us to monitor molecular alterations because of its non-invasiveness. The detection rate is higher in patients with distant metastases than in those with disease limited to the intrathoracic region. The results are not always correlated with treatment efficacy because inter-tumor heterogeneity exists, especially after acquired resistance. Re-biopsy is invasive and difficult to perform repeatedly. Results differ depending on the site to be collected due to tumor heterogeneity. However, we can focus re-biopsy on target lesions causing symptoms. In addition, it is possible to analyze, not only T790M mutation but also tissue change, such as transformation to small cell carcinoma (26), which is one of the *EGFR*-TKI resistance mechanisms. Considering these characteristics, a combination of liquid biopsy and re-biopsy should contribute to enhanced treatment. Hereafter, combining liquid biopsy and re-biopsy with spatial molecular diagnosis using imaging should prove even more beneficial.

Funding

This work was supported by JSPS KAKENHI [Grant Number JP17K07197] to [T.N. and N.S.-A.].

Conflicts of Interest

Shinya Kimura received the scholarship donation from Chugai. All other authors have no conflicts of interest to declare.

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Received April 5, 2018

Revised April 29, 2018

Accepted May 7, 2018